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A SAMPLING METHOD FOR SOLID SUBSTANCES IN HIGH-TEMPERATURE GAS CHROMATOGRAPHY UP TO 500°

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(Received October 5th, 1959)

Sampling in gas chromatography is one of the important problems of practical analysis when it is necessary to measure small or minute quantities of substances or their mixtures. In principle, the mixture of substances is sampled in the gaseous state (gases, vapours) or in the liquid state (liquids, solids in solutions, or melts).

Sampling of gaseous substances[1–15] is relatively the easier problem. In all the methods used sampling is carried out with a gas burette (*e.g.*[1]), or a pipette (*e.g.*[3]). The individual modifications differ only in their form. For the introduction of a liquid sample a pipette[5,16–27] (see also[31]) or an injection syringe[28–30] are used. These methods allow easy manipulation of even small volumes of liquids, but a precise measurement of the sample is rarely possible so that we are always limited to methods of indirect computation (by means of an internal standard; assuming that the sum of all peak areas equals 100 %). Sampling of solids has up to the present been performed, either in the melted state or in solution. In methods requiring precise quantities the substance is weighed into glass capillary tubes and crushers are used to break the tubes[31]. Sampling in the case of capillary columns where it is necessary to take a hundredth part or possibly even a smaller part from the sample introduced by some of the above-mentioned ways, presents a special problem. A system of two capillary tubes or a valve in T-form are used for this purpose[39,40].

The simplest and most common sampling system is that of Ray[28], in which a syringe is used; by this method it is possible to analyse gases and liquids up to temperatures of 200–250° without any special complications. The sampling pipettes, which are furnished with stopcocks, are suitable for temperatures of 150–200°. If glass or metal stopcocks are used, the lack of gas tightness already plays a role at temperatures of 150°; stopcocks of synthetic resistant materials (Teflon) on the other hand, are only stable to heating up to a temperature of 200° at most. The same holds for sampling by means of a capillary tube on the end of a supporting rod which can be inserted in the sampling device[18]. The glass capillary tube crushers are always constructed on a mechanical principle. The use of mechanical crushers is limited again up to the temperature range of 250–300° due to the packing and temperature stability problems arising with the materials that can be used.

For sampling an exactly known quantity of a substance, mainly in the solid state, and for sampling at temperatures of 150–500°, *i.e.* in the field of gas chromatography at elevated to very high temperatures, where the sampling methods hitherto described do not give satisfactory results, the methods using metal capillary tubes made of easily fusible alloys have proved useful in our work.

EXPERIMENTS AND RESULTS

A sample of a solid (or a liquid) substance is weighed into a capillary container made from a lamella of an easily fusible alloy.

The lamella is obtained by allowing Wood's metal (melting point: 60.5°)* to trickle onto a cold glass plate. An oblong piece of 8 × 10 mm is cut out. By rolling this on a metal core, a small tube of inner diameter of about 2 mm is formed, which is soldered by touching the bottom and the side with a warm object. The weighed sample is introduced into the capillary tube and this is then closed by soldering in the same way.

The capillary tube with the weighed sample is dropped through the open metal stopcock 1 (Fig. 1) into the cold inlet of the sampling device which is connected to the metal chromatographic column. During the opening of stopcock 1, an addition-

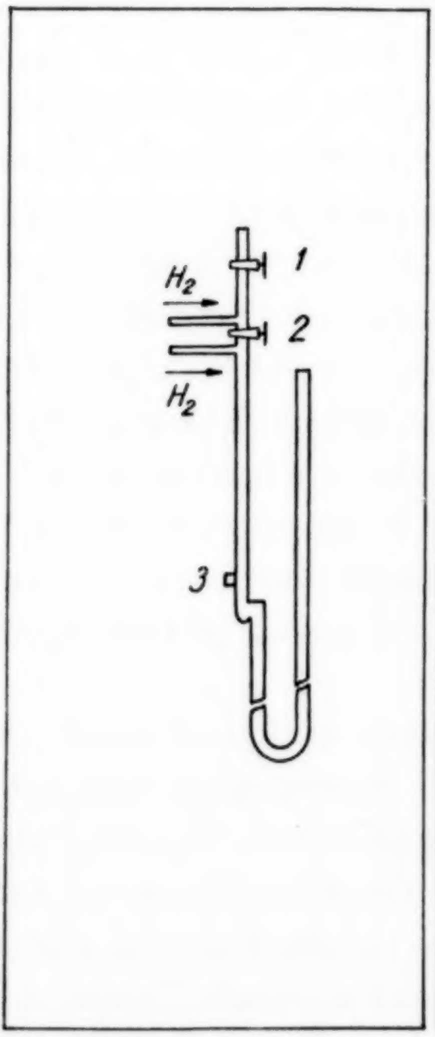

Fig. 1. Scheme of the chromatographic column with the sampling nipple.

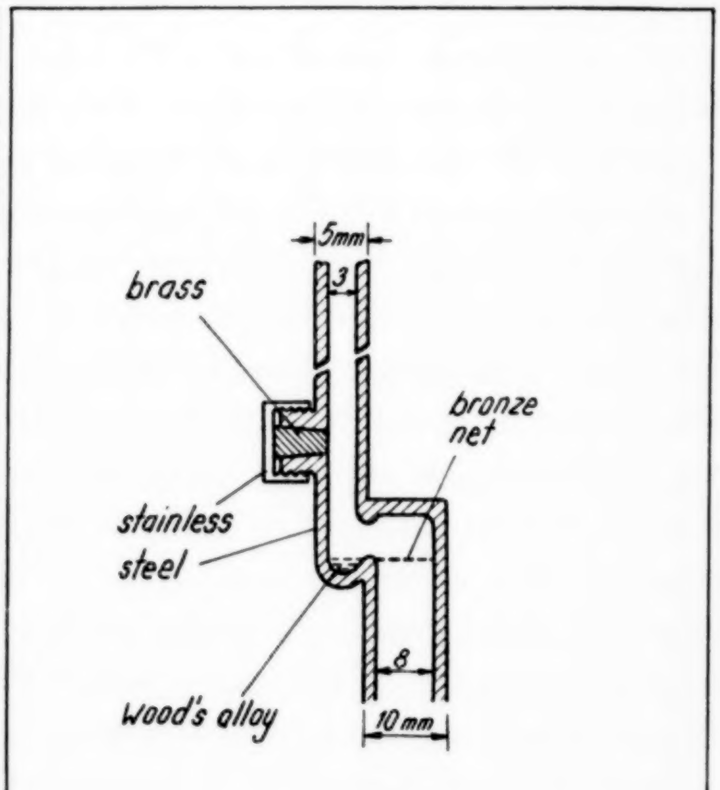

Fig. 2. Detail of the vaporizing saddle.

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* Since the material used to make the sampling capillary tubes is not lost, it is possible to use even more costly alloys with lower melting points (*e.g.*: 10.6 % Sn, 22.1 % Pb, 41.0 % Bi, 8.2 % Cd and 18.1 % In, which has a melting point of 47°).

al stream of carrier gas flows through the open cold inlet, thus preventing air from entering the sampling device. After stopcock 1 has been closed, a metal capillary tube is dropped into the vaporizing part, which is a saddle-shaped tube (Fig. 2). The container that has been dropped in melts and the liberated sample is carried with the carrier gas into the chromatographic column. A minute quantity of air, or inert gas enclosed in the capillary tube, indicates in the chromatogram the time necessary for the sample to pass through the free area in the column (t_0). The specific gravity of the

TABLE I

COURSE OF MELTING OF THE CAPILLARY SAMPLING TUBE (m.p. 60°) IN RELATION TO THE TEMPERATURE

Saddle temp. °C	*Melting time*	
	Beginning sec	*Completion* sec
140	0.8	3
150	0.4	1
175	0.2	0.4
250	instantaneously	< 0.4
310	instantaneously	< 0.3
410	instantaneously	< 0.2

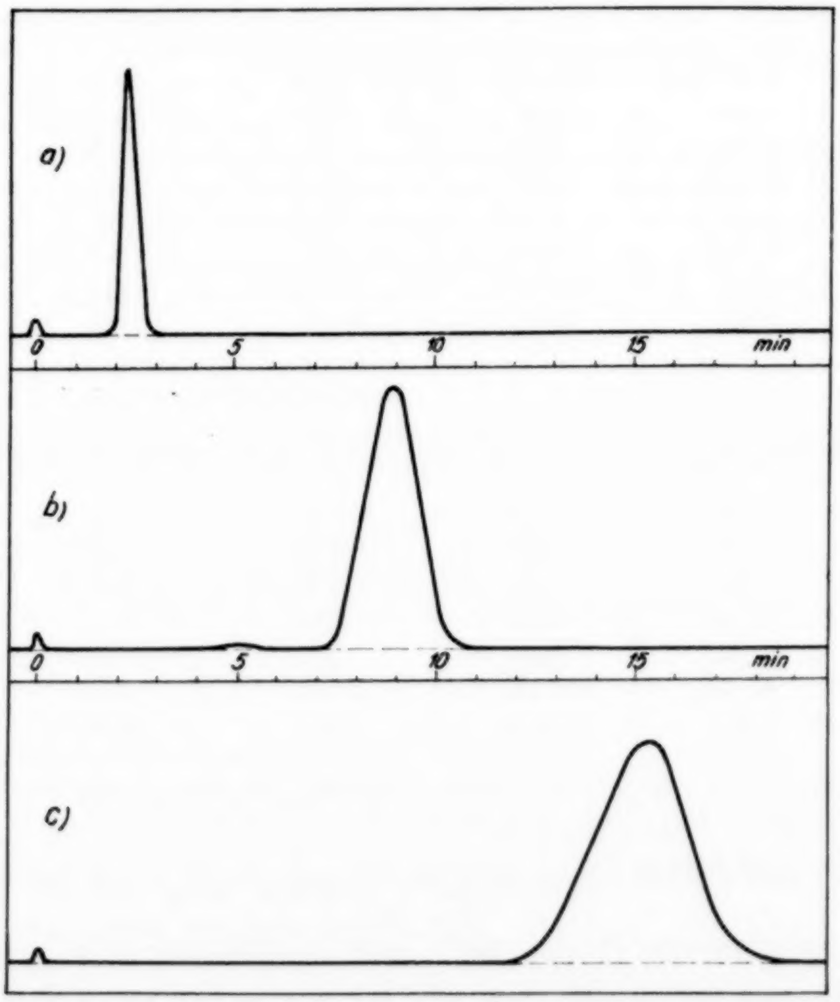

Fig. 3. Chromatograms of samples introduced by means of metal capillary tubes (column 16.2 g Celite 545, impregnated with 20 wt.-% silicone elastomer E 301; temp. 192°; hydrogen as carrier gas; detection with a catharometer). (a) 2.02 mg 3,4-dimethylphenol (m.p. 62.5°, b.p. 226°). (b) 4.23 mg 2,3-dimethylnaphthalene (m.p. 104.5°, b.p. 269°). (c) 4.13 mg fluorene (m.p. 115°, b.p. 295°).

metal and the surrounding temperature are sufficient to guarantee that the sample is not enclosed in the melted alloy. The quantity of metal in the lamella is very small (50 to 100 mg) and therefore it is possible to perform series of up to 200 analyses without emptying the sampling saddle. After a larger series the sampling saddle is emptied by allowing the melted alloy to flow away through a special orifice 3 (Fig. 1).

When verifying the procedure, the time and temperature required to melt the capillary tube and vaporize the sample were first studied. From Table I it can be seen that already at temperatures above 150° the period during which melting of the whole capillary tube made of Wood's metal is completed is acceptable for gas chromatographic determinations. At temperatures above 250° liberation of the sample from the capillary tube is practically instantaneous.

In Fig. 3 examples are given of practical chromatograms of substances with boiling points that are even 100° higher than the temperature of the evaporating saddle.

Whether the evaporation was quantitative was studied by measuring the peak areas (with a planimeter) of various weighed samples. In Table II examples of repeated measurements of naphthalene (b.p. 218°) and resorcinol (b.p. 276°) are presented. The average error of one determination is within the limits of the relative error of a planimetric determination of the peak area[41].

TABLE II

REPRODUCIBILITY OF PEAK AREAS

Sample *mg*	*Area in mm²*		*Relative error* *%*
	found	*interpol.**	
Naphthalene (m.p. 80.2°, b.p. 218°); column temperature 192°			
0.88	320	303	3.0
1.72	580	610	4.9
2.19	811	775	4.6
2.40	876	840	4.3
2.56	915	890	2.7
2.68	913	930	1.8
3.05	1070	1055	1.4
3.45	1151	1190	3.6
		average error =	3.7
Resorcinol (m.p. 110.7°, b.p. 276°); column temperature 204°			
1.18	355	350	1.4
1.66	480	510	5.9
1.83	590	580	1.7
2.59	850	850	0.0
2.94	965	970	0.5
3.51	1170	1165	0.4
3.61	1200	1200	0.0
		average error =	2.5

* Obtained from the calibration curve plotted through experimental points, by interpolation.

The use of metal capillary tubes is limited to those substances that do not react with the metal of the capillary tube and are not attacked by it.

SUMMARY

A sampling method for solids (or liquids) using metal capillary tubes made of fusible alloys was devised. The method is already very suitable at a temperature of 150°, and has proved to be more useful than the procedures hitherto described, especially for high-temperature gas chromatography up to 500°.

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CHROMATOGRAPHIC SEPARATION OF DERIVATIVES OF PHENOL ON CATION EXCHANGE RESINS

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It has been recognized by many investigators that aromatic compounds are strongly adsorbed on synthetic ion exchange resins[1-8] and this phenomenon has been utilized for the chromatographic separation of phenol and its derivatives using a column of cation exchange resins[9-11].

Since this method (*i.e.* the use of cation exchange resins as the adsorbents for aromatic substances) proved to be very effective for the chromatographic separation of DNP-amines[12], of phenolic acids[13], of DNP-amino acids[14] and even of lower fatty acids[15], the chromatographic conditions used in the earlier study were reinvestigated and we succeeded in separating chromatographically the three isomers of cresol and dihydroxybenzene using Amberlite IR 112 or Duolite C 25 as the adsorbent and alkaline (pH 9.70 ± 0.02) or neutral (pH 4.00 ± 0.02) buffers as eluents. The separation of hydroquinone, pyrocatechol, phenol, *p*-cresol and *p-tert.*-butylphenol on a column of partially esterified Amberlite IRC 50 [16] was achieved with the use of a mixture of ethanol, methanol and 0.1 *N* hydrochloric acid (3:1:4 by vol.) as eluent.

EXPERIMENTAL

Materials

2,6-Dinitrophenol was prepared by the method of HÜBNER AND SCHNEIDER[17]. *p-tert.*-Butylphenol was prepared according to the method of LIEBMANN[18]. Other compounds used were commercial products. Citric acid monohydrate, sodium hydrogen carbonate, sodium carbonate (anhydrous), sodium hydroxide and benzyl alcohol were special grade. Ethanol (99 %), methanol and methyl ethyl ketone were distilled before use.

Ion exchange resins

Amberlite IR 112 (A.G. Na^+ form) was pulverized in a mortar and passed through a 150 mesh screen with running water and then filtered through a 240 mesh screen in order to remove fine particles. The filtered resin was washed with 6 *N* hydrochloric acid and 2 *N* sodium hydroxide solution[19]. Duolite C 25 was pulverized and washed as described previously (200 to 300 mesh)[13]. Amberlite IRC 50 (A.G.) was pulverized and washed as described previously (200 to 300 mesh)[15]. It was partially esterified by refluxing with aqueous acidic alcohol in the following way: about 60 ml of H form Amberlite IRC 50 was mixed with 600 ml of a mixture of ethanol, methanol and *N* hydrochloric acid (6:2:5 by vol.) and boiled under reflux for 40 h. The bumping of the mixture could be prevented by adding two or three pieces of porcelain boiling stone (8 mm cube), the edges of which were removed by grinding.

Preparation of the chromatographic column

The resin was equilibrated on a glass filter with the solvent to be used for the chromatography (Table I) and suspended by swirling in about two volumes of the same solvent. The suspension was poured into a chromatographic tube through a small funnel fitted with a ground joint and allowed to settle[15]. After about 50 ml of the solvent has been passed through, the column is ready for use.

TABLE I

COMPOSITION OF THE SOLVENTS

Solvent	*Composition*
A	25 ml of benzyl alcohol was dissolved in 1 l of pH 4.0 buffer*.
B	250 ml of methyl ethyl ketone was dissolved in 1 l of pH 4.0 buffer*.
C	250 ml of ethanol was dissolved in 1 l of pH 4.0 buffer*.
D	methanol–ethanol–0.1 *N* HCl (1:3:4 by vol.).
E	9.3 g of sodium hydrogen carbonate, 8.5 g of anhydrous sodium carbonate and 0.5 g of EDTA (disodium salt) were dissolved in distilled water and made up to 1 l (pH 9.70 ± 0.02).
F	4.2 g of sodium hydrogen carbonate, 3.1 g of anhydrous sodium carbonate and 0.25 g of EDTA (disodium salt) were dissolved in distilled water and made up to 1 l (pH 9.65 ± 0.02). Then 200 ml of ethanol was added.

* pH 4.0 buffer was made as follows: 21.0 g of citric acid monohydrate, 6.0 g of sodium hydroxide were dissolved in distilled water, the pH was adjusted to 4.00 ± 0.02 with 2 *N* hydrochloric acid and the solution was made up to 1 l with distilled water.

Chromatographic separation and quantitative analysis of synthetic mixtures

A mixture of the phenolic compounds to be analyzed was dissolved in the solvent to be used for chromatography (Table II) and 1.0 ml of the solution was added to the column. The effluent was collected in fractions of 40 drops in test tubes with a graduation mark at 3.5 ml. Quantitative analyses were performed spectrophotometrically. The solvent used for the chromatographic separation was added to the fractions

TABLE II

CONDITIONS OF THE CHROMATOGRAPHIC SEPARATION

Figure	*Resin*	*Form of the resin*	*Size of the column (cm)*	*Solvent*	*Room temperature (°C)*
1	Amberlite IR 112	Na^+	0.8 × 64	A	27–30
2	Duolite C 25	Na^+	0.7 × 143	B	27–30
3	Duolite C 25	Na^+	0.7 × 143	C	27–30
4	Amberlite IRC 50	H	0.8 × 112	D	21–23
5	Amberlite IR 112	Na^+	0.8 × 150	E	30–32
6	Duolite C 25	Na^+	0.7 × 143	F	29–31

TABLE III

PER CENT RECOVERY OF DERIVATIVES OF PHENOL FROM THE CHROMATOGRAPHIC COLUMNS

Derivatives of phenol	*Wave length used for the assay (mμ)*	*Solvent*					
		A	*B*	*C*	*D*	*E*	*F*
Picric acid	360		100				
2,6-Dinitrophenol	435		96				
2,4-Dinitrophenol	360	106	96				
o-Nitrophenol	420	71	82				
m-Nitrophenol	395	96					
p-Nitrophenol	405	106					
Hydroquinone	290			100	97		
Resorcinol	274			101			
Pyrocatechol	276			88	97		
Phenol	288				98	98	88
o-Cresol	290					98	
m-Cresol	290					98	96
p-Cresol	297				95	95	
p-tert.-Butylphenol	296				90		

containing dihydroxy compounds as a diluent to give 3.5 ml, and *N*/2 sodium hydroxide solution was added to the fractions containing phenol, cresols and *p-tert.*-butylphenol as a diluent to give 3.5 ml. 10% sodium carbonate solution was added to the fractions containing nitrophenols as a diluent to give 3.5 ml. The absorbance of each fraction was measured at the wave lengths indicated in Table III using a Beckman model DU quartz spectrophotometer and diluting the fractions of high optical density two or three fold with the diluent described above.

RESULTS AND DISCUSSION

As shown in Figs. 1 and 2, nitrophenols were separated on columns of Amberlite IR 112 and Duolite C 25. Since the polynitrophenols examined have a negative charge at the pH of eluent A and B, and the resins have a negative charge, polynitrophenols were excluded from the resin phase and eluted faster than the mononitrophenols[9]. Mononitrophenols were adsorbed in an undissociated form and the separation seemed to be effected by non-ionic adsorption. The same will be true for the separation of dihydroxybenzenes on a column of Duolite C 25 and for the separation of dihydroxybenzenes, phenol and alkylated phenols on a column of Amberlite IRC 50 (Figs. 3 and 4).

Ortho-, *meta-* and *para*-cresol were separated successfully on a column of Amberlite IR 112 using solvent E (Fig. 5). When Duolite C 25 was used as an adsorbent, the separation of *o*- and *p*-cresol was unsatisfactory, although *m*-cresol could be separated from other cresols (Fig. 6). Since the three isomers of cresol could not be separated using an eluent of lower pH (*e.g.* pH 4.0), the separation achieved must be due to the difference between the p*K* value of the phenolic hydroxyl group of the isomers of cresol[20] and the position of the methyl group relative to the phenolic hydroxyl group. That both

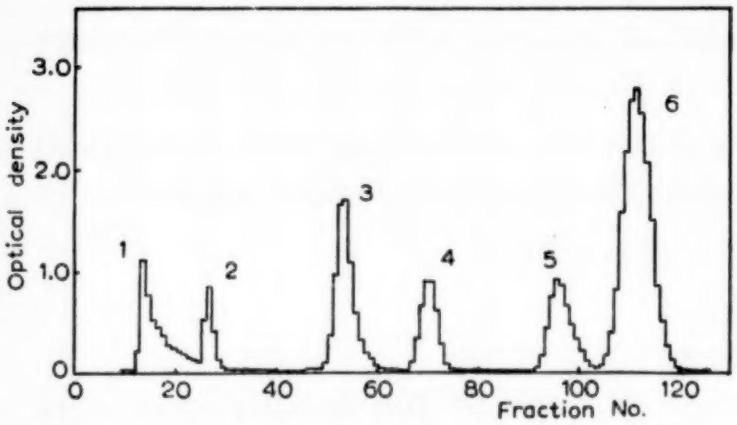

Fig. 1. Elution curve of nitrophenols. Optical density was measured at 360 mμ (fraction 10–24), at 435 mμ (fraction 25–46), at 360 mμ (fraction 47–63), at 420 mμ (fraction 64–80), at 395 mμ (fraction 81–104) and at 405 mμ (fraction 105–126). The compounds in the order of their elution from the column, are: picric acid (1), 2,6-dinitrophenol (2), 2,4-dinitrophenol (3), *o*-nitrophenol (4), *m*-nitrophenol (5) and *p*-nitrophenol (6).

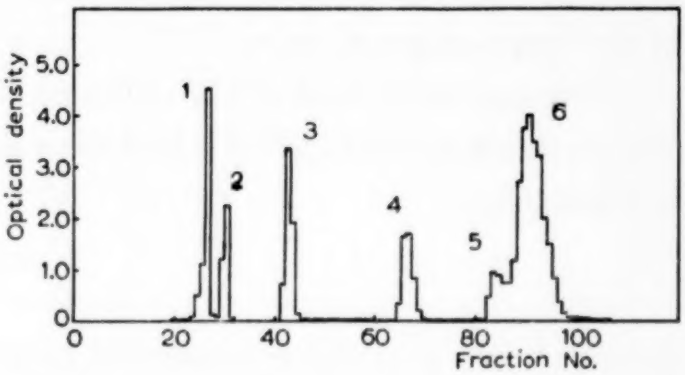

Fig. 2. Elution curve of nitrophenols. Optical density was measured at 360 mμ (fraction 20–28), at 435 mμ (fraction 29–38), at 360 mμ (fraction 39–60), at 420 mμ (fraction 61–78) and 395 mμ (fraction 79–107). The compounds in the order of their elution from the column, are: picric acid (1), 2,6-dinitrophenol (2), 2,4-dinitrophenol (3), *o*-nitrophenol (4), *m*-nitrophenol (5) and *p*-nitrophenol (6).

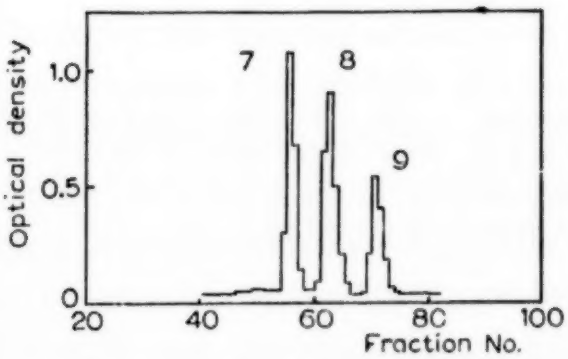

Fig. 3. Elution curve of the three isomers of dihydroxybenzene. Optical density was measured at 290 mμ (fraction 41–60), at 274 mμ (fraction 61–67) and at 276 mμ (fraction 68–82). The compounds in the order of their elution from the column, are: hydroquinone (7), resorcinol (8) and pyrocatechol (9). Phenol overlapped pyrocatechol.

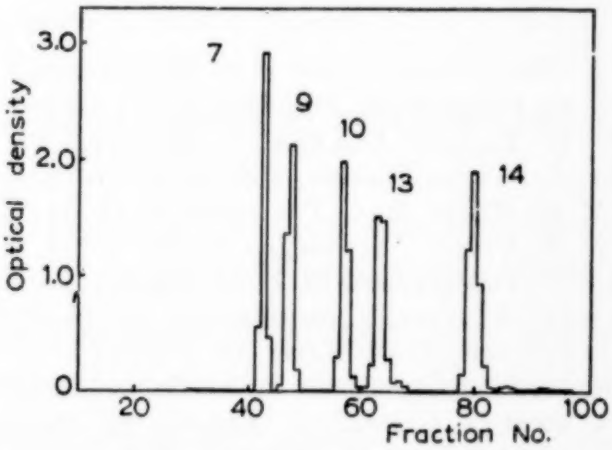

Fig. 4. Elution curve of dihydroxybenzenes, phenol and alkylphenols. Optical density was measured at 290 mμ (fraction 30–45), at 276 mμ (fraction 46–54), at 288 mμ (fraction 55–60) and 296 mμ (fraction 61–97). The compounds in the order of their elution from the column, are: hydroquinone (7), pyrocatechol (9), phenol (10), *p*-cresol (13) and *p*-*tert*.-butylphenol (14).

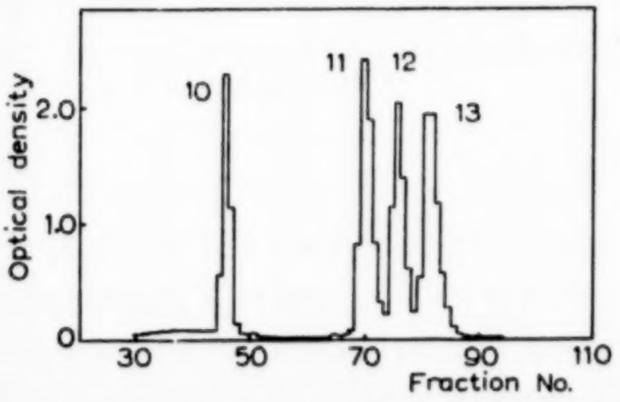

Fig. 5. Elution curve of phenol and the three isomers of cresol. Optical density was measured at 288 mμ (fraction 30–60), at 290 mμ (fraction 61–79) and 297 mμ (fraction 80–95). The compounds in the order of their elution from the column, are: phenol (10), *m*-cresol (11), *o*-cresol (12) and *p*-cresol (13).

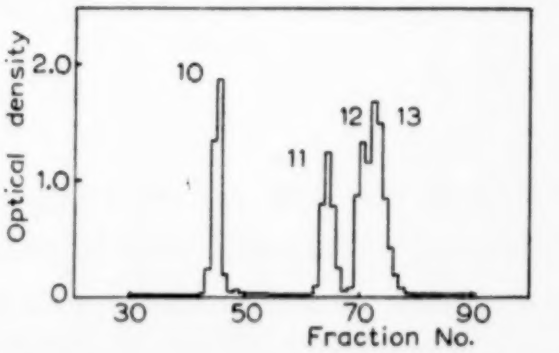

Fig. 6. Elution curve of phenol and the three isomers of cresol. Optical density was measured at 288 mμ (fraction 30–59) and at 290 mμ (fraction 60–91). The compounds in the order of their elution from the column, are: phenol (10), *m*-cresol (11), *o*-cresol (12), and *p*-cresol (13).

effects are operative could be seen from the elution sequence which is not in the order of the increasing pK value.

The optimum load of the columns used was 0.5 to 2 mg/cm^2 per each component and as much as 10 mg/cm^2 could be added when there was an efficient separation of components.

SUMMARY

It was shown that three isomers of mononitrophenol and of dihydroxybenzene were separated on a column of Amberlite IR 112 or of Duolite C 25 using solvent A or C. Separation of polynitrophenols was successful on a column of Duolite C 25 using solvent B. Isomers of cresol were separated from each other on a column of Amberlite IR 112 using solvent E and *m*-cresol was separated from other cresols on a column of Duolite C 25 using solvent F. Hydroquinone, pyrocatechol, phenol, *p*-cresol and *p-tert.*-butylphenol could be separated on a column of partially esterified Amberlite IRC 50 using solvent D.

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RETENTION TIMES IN PROGRAMMED TEMPERATURE GAS CHROMATOGRAPHY*

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An important development in analysis is the method of programmed temperature gas chromatography[1,2]. By this method the column temperature is continually increased so that the lighter fractions are eluted at low temperatures and the heavier fractions at high temperatures. Each fraction is eluted at a temperature near the optimum temperature for that fraction alone. Since a large temperature range can be covered, a wide b.p. range of solutes can be eluted in one experiment in a convenient time. The method is conceptually simple and at the same time capable of bringing wide b.p. fractions within the confines of a single experiment.

The theoretical analysis presented here is intended to show the influence of molecular structure, heating rate, gas velocity, etc., on the retention times of various solutes. As is known from experimental work, the continual linear increase of temperature during a run leads to the elution of uniformly spaced peaks in a homologous series such that the retention time–number of carbon atoms plot is roughly linear[2]. Isothermally, the retention time increases nearly exponentially with the number of carbon atoms. This requires excessive waiting times for the higher members. The nearly linear relationship of the programmed temperature method is a consequence of the fact that the retention time increases approximately linearly with the free energy difference between solute in the gas phase and solute in the stationary phase (both in a standard state). The conditions under which the linear approximation is reasonably good will be investigated.

The differential equation describing the position s of the center of a zone is

$$\frac{\mathrm{d}s}{\mathrm{d}t} = R_F(t)v(s,t) \qquad (1)$$

where R_F is the ratio of zone velocity to carrier gas velocity, and is a function of temperature T which in turn is a controlled function of time. The carrier gas velocity v is a controlled function of time and, in addition, is a function of distance if the pressure drop in the column is large. The dependence upon distance can be established from the theory of MARTIN AND JAMES[3] providing the column dead time (passage time for inert molecules) is small compared to the time in which significant fractional changes in pressure or absolute temperature occur. If the temperature change is abnormally

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rapid, the velocity will be significantly influenced by expansion within the column. Fortunately, experiments rarely occur outside this limit since the column dead time is of the order of 20 sec.

With the above assumptions the gas velocity becomes

$$v = \frac{v_0}{\sqrt{1 + \frac{2v_0}{cp_0}(L - z)}} \tag{2}$$

where z is the distance measured from the inlet of a column of length L, p_0 and v_0 are the values of pressure and velocity at the outlet, and c is defined by the following relationship,

$$c\frac{\mathrm{d}p}{\mathrm{d}z} = v \tag{3}$$

and is dependent upon column packing and gas viscosity, but is independent of pressure. Since c is dependent upon the coefficient of viscosity, it also is dependent upon temperature and time. This fact makes the integration of eqn. (1) difficult when eqn. (2) is substituted into it. For this reason we will assume that the pressure difference from outlet to inlet remains small compared to either, and that minor corrections can be made by choosing a suitable average velocity.

An expression for R_F, also needed in eqn. (1), will now be obtained. The equilibrium ratio of the number of moles in the mobile phase to the number in the stationary phase is

$$\frac{R_F}{1 - R_F} = \frac{A_m}{A_s} \mathrm{e}^{-\Delta F^0/RT} \tag{4}$$

where the solute is assumed to form a Henry's law solution, and the gas mixture is ideal. The standard state for the free energies are both in the same concentration units. The standard free energy change from the stationary to the mobile phase is ΔF^0. The ratio of mobile phase to stationary phase cross sectional areas is A_m/A_s. From this we obtain

$$R_F = \frac{(A_m/A_s)\,\mathrm{e}^{-\Delta F^0/RT}}{1 + (A_m/A_s)\,\mathrm{e}^{-\Delta F^0/RT}} \tag{5}$$

Since R_F is usually of the order of 10^{-2}, except perhaps right at the moment of elution, and since eqn. (5) is difficult to treat as such, we make the following approximation

$$R_F = \frac{A_m}{A_s} \mathrm{e}^{-\Delta F^0/RT} \tag{6}$$

Eqn. (1) is now integrated along the column length. From this we obtain

$$L = \int_0^{t_r} R_F v \,\mathrm{d}t \tag{7}$$

where t_r is the retention time. This equation is the starting point from which retention times are obtained irrespective of the particular approximations made. In our case we assume v to be constant and R_F to be given by eqn. (6). Furthermore we assume a linear increase in temperature, $T = T_0 + \beta t$, in accordance with common experimental practice. Also, ΔH^0 and ΔS^0, the standard enthalpy and entropy changes, are assumed to be independent of temperature.

$$L = \frac{A_m v\, e^{\Delta S^0/R}}{A_s \beta} \int_{T_0}^{T_r} e^{-\Delta H^0/RT}\, dT \qquad (8)$$

Eqn. (8) involves an integration over temperature where T_r is the retention temperature, or the temperature at which the peak appears. If desired t_r can be obtained from T_r.

If the variable of integration is changed to $X = \Delta H^0/RT$, we obtain,

$$\frac{R\beta t^0}{\alpha \Delta H^0} = \int_{X_r}^{X_0} \frac{e^{-X}}{X^2}\, dX \qquad (9)$$

where $\alpha = (A_m/A_s)\, e^{\Delta s^0/R}$ and $L/v = t^0$, the column dead time. Integrating by parts, we find,

$$\frac{R\beta t^0}{\alpha \Delta H^0} = \frac{e^{-X_r}}{X_r} + E(-X_r) - \frac{e^{-X_0}}{X_0} - E(-X_0) \qquad (10)$$

where $E(X)$ is the exponential integral and

$$-E(-X) = \int_X^{\infty} \frac{e^{-X}}{X}\, dX \qquad (11)$$

the value of this integral can be acquired from standard tables. One then obtains X_r by numerical means from eqn. (10), and thence T_r from $T_r = \Delta H^0/RX_r$.

A somewhat simpler approximate equation can be obtained by using the fact that e^{-X} is a more rapidly varying function than $1/X^2$ when $X > 2$. Hence in this region

$$\int_X^{\infty} \frac{e^{-X}}{X^2}\, dX \cong \frac{1}{(X+1)^2} \int_X^{\infty} e^{-X}\, dX = \frac{e^{-X}}{(X+1)^2} \qquad (12)$$

where $1/X^2$ has been assumed constant for the integration and has been assigned the value it has at the center of gravity $(X + 1)$ of the remaining integral (the approximation, e^{-X}/X^2, to the integral has also been tried, but it is not found to be so satisfactory). Since X_0 and X_r are ordinarily of magnitude five or larger, the approximation is quite good. Using this approximation in combination with eqn. (9) we obtain

$$\frac{R\beta t^0}{\alpha \Delta H^0} = \frac{e^{-X_r}}{(X_r+1)^2} - \frac{e^{-X_0}}{(X_0+1)^2} \qquad (13)$$

For retention temperatures large compared to the starting temperature T_0, the term containing $X_0 = \Delta H^0/RT_0$ is negligible, and we have

$$\frac{R\beta t^0}{\alpha \Delta H^0} = \frac{e^{-X_r}}{(X_r+1)^2} \qquad (14)$$

It is unfortunate that none of the equations from which T_r is obtained provides a direct solution in terms of T_r. This quantity must be obtained by numerical means although this is quite simple using eqn. (14) or (13). A good deal of approximate information can be obtained directly from the fact that X_r is considerably larger than unity. It is not, unfortunately, sufficiently large that X_r can be substituted for $X_r + 1$ in eqn. (14) without some effect (the approximation is fair, however). Likewise we cannot state the variation in $1/(X_r + 1)^2$ is completely negligible compared to the variation in e^{-X_r} for a given variation in X_r. However this also is a fair approximation over a limited range. If we make the assumption that changes in ΔH^0 and T_r are reflected most strongly in the term e^{-X_r} we are led to the conclusion that X_r is a constant for a given run. Consequently $T_r = (\text{const}) \times \Delta H^0$. If ΔH^0 increases linearly with the number of carbon atoms, then T_r (and also t_r) likewise increases linearly with the number of carbon atoms. The nearly linear increase of T_r with carbon atom number has been observed[2], but the departure from linearity is reproducible and real. This matter will be discussed shortly with entropy effects also accounted for.

VARIATION OF PARAMETERS

The next topic to be dealt with is the effect of small changes in various parameters upon the retention temperature. This is best done by defining the following function f related to eqn. (8)

$$f = \int_{T_0}^{T_r} e^{-\Delta H^0/RT_r}\, dT - g = 0 \tag{15}$$

where

$$g = \frac{A_s t^0 \beta\, e^{-\Delta S^0/R}}{A_m}$$

Variations may occur in T_r, ΔH^0 and one of the several variables in g. Differentiating eqn. (15)

$$df = \frac{\partial f}{\partial T_r} dT_r + \frac{\partial f}{\partial \Delta H^0} d\Delta H^0 + \frac{\partial f}{\partial g} dg = 0 \tag{16}$$

where

$$\frac{\partial f}{\partial T_r} = e^{-\Delta H^0/RT_r}, \quad \frac{\partial f}{\partial g} = -1$$

$$\frac{\partial f}{\partial \Delta H^0} = \frac{-1}{R} \int_{X_r}^{X_0} \frac{e^{-X}}{X} dX \cong -\frac{1}{R}\left(\frac{e^{-X_r}}{X_r + 1} - \frac{e^{-X_0}}{X_0 + 1}\right) \tag{17}$$

The last approximation is of the same kind (except perhaps better) as that made in eqn. (12). From eqns. (16) and (17) we find that

$$\frac{\partial T_r}{\partial \Delta H^0} = \frac{T_r}{\Delta H^0 + RT_r} - \frac{T_0\, e^{X_r - X_0}}{\Delta H^0 + RT_0} \cong \frac{T_r}{\Delta H^0 + RT_r} \tag{18}$$

the last approximation being good at high retention temperatures. Making the assumption that $X_r \gg 1$ ($\Delta H^0 \gg RT_r$), which is a "fair" approximation, eqn. (18) integrates immediately to yield a straight line relationship between T_r and ΔH^0. If a tangent is drawn to this "nearly" straight line, it intercepts the temperature axis at $T_1 = T_r/(X_r + 1)$. This temperature is usually well within 100° of absolute zero.

Further use of eqns. (16) and (17) shows that

$$\frac{\partial T_r}{\partial g} = e^{\Delta H^0/RT_r} = e^{X_r} \tag{19}$$

A change in g might reflect a change in the heating rate β, the column dead time t^0, the standard entropy change ΔS^0, or A_s, which is nearly proportional to the weight percent of the stationary liquid. The following equations apply to these cases

$$\frac{\partial T_r}{\partial \beta} = \frac{t^0}{\alpha}\,\mathrm{e}^{X_r} = \frac{A_s t^0}{A_m}\,\mathrm{e}^{\Delta F^0/RT_r} \tag{20a}$$

$$\frac{\partial T_r}{\partial t^0} = \frac{\beta}{\alpha}\,\mathrm{e}^{X_r} = \frac{A_s \beta}{A_m}\,\mathrm{e}^{\Delta F^0/RT_r} \tag{20b}$$

$$\frac{\partial T_r}{\partial A_s} = \frac{t^0 \beta}{A_m}\,\mathrm{e}^{\Delta F^0/RT_r} \tag{20c}$$

$$\frac{\partial T_r}{\partial \Delta S^0} = \frac{-g}{R}\,\mathrm{e}^{X_r} = -\,\frac{t^0 \beta A_s}{R A_m}\,\mathrm{e}^{\Delta F^0/RT_r} \tag{20d}$$

RESULTS

Calculations have been made which illustrate the application of the various equations and show how experimental results may be related to theory. First we show, in Fig. 1 a comparison of the exact integration of eqn. (9) as shown in eqn. (10), to the approximate integration as summarized by eqn. (13). We see that the approximation is very satisfactory, and shall consequently use it for the remainder of the calculations. The heating rate β has been given the value 0.5°/sec or 30°/min. The approximation improves somewhat at lower heating rates (since X_r is larger), and decreases in value at higher heating rates.

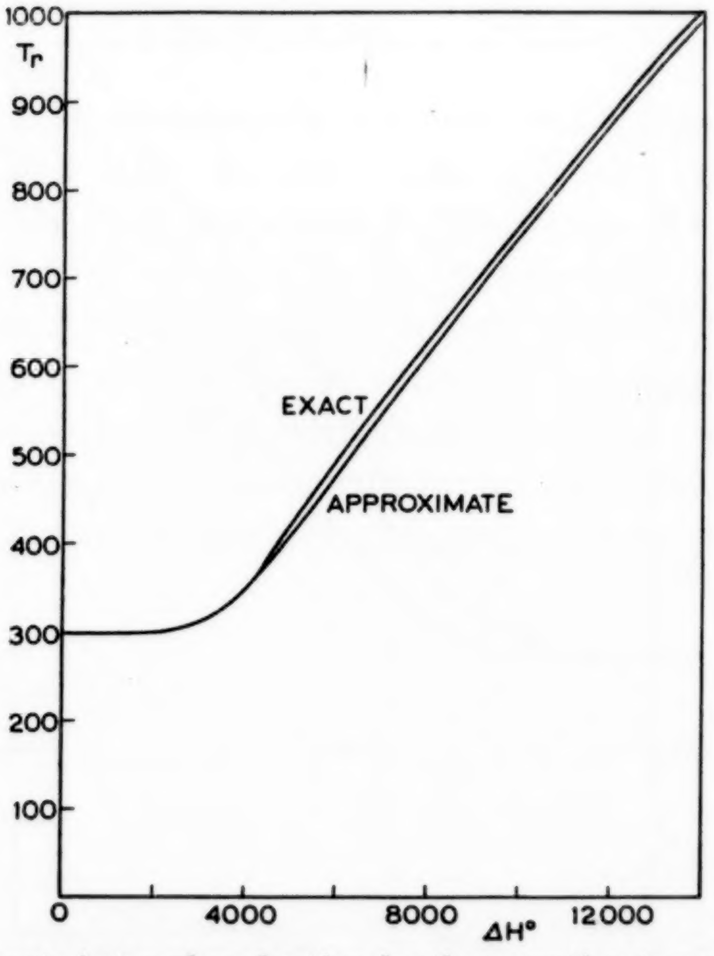

Fig. 1. Exact and approximate integral evaluation for the retention temperature, T_r, with $\beta = 0.5$.

Other parameters have been selected as follows. The values quoted will be used hereafter unless otherwise noted. The column dead time t^0 is arbitrarily set at 20 sec. This of course varies over wide limits depending on velocity and length. The initial temperature T_0 is 300° K. The ratio $a = A_m \, e^{\Delta s^0/R}/A_s$ is given the value of 100. This corresponds to an A_m/A_s ratio of 3 to 5, and a ΔS^0 value of 6 or 7 entropy units. The positive entropy reflects the increase in randomness upon escaping from the somewhat "ordered" structure of the liquid. A rough estimate of its value is gained by: (1) Using Trouton's rule entropies, and assuming the solute to form an ideal solution upon dilution with the solvent; (2) by assuming the known solubility of inert gases (especially helium) in non-polar liquids to be governed by entropy effects; and (3) by extrapolating curves[4] in which the log of the retention volume is plotted against the reciprocal temperature to zero. The latter method gives values several units larger than those quoted. This difference may arise, in part, due to the wide range needed for the extrapolation.

It must be emphasized that the arbitrary selection of parameters will permit showing only the approximate interdependence of variables, and that each case must be treated individually for exact values.

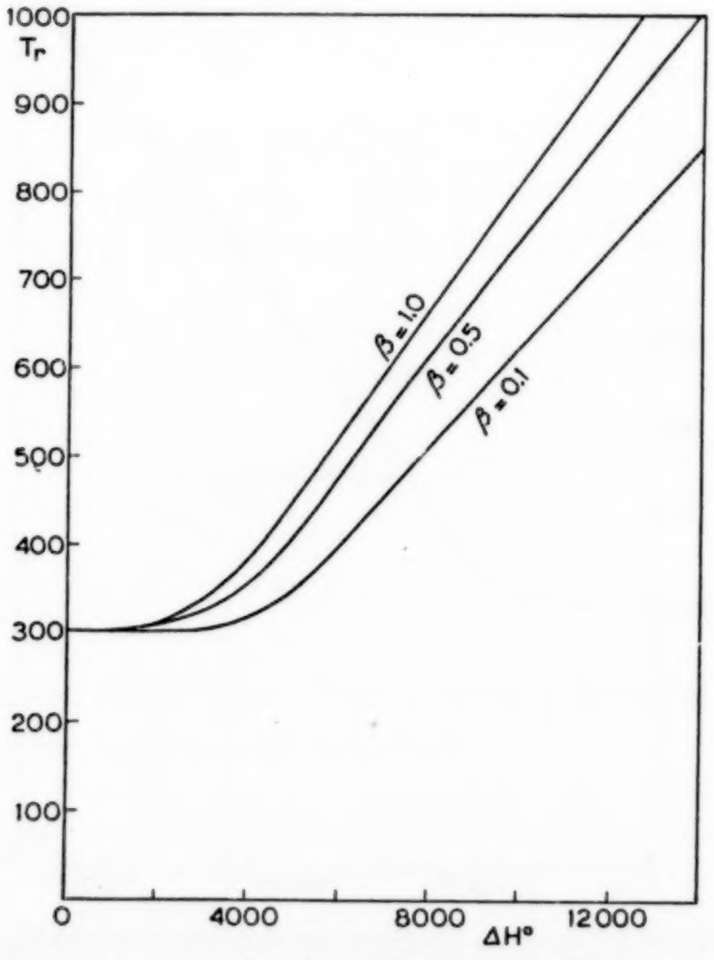

Fig. 2. Retention temperature curves for the different heating rates, β = 1.0, 0.5, 0.1°/sec.

Fig. 2 shows the retention temperature curves for three different heating rates, β = 1.0, 0.5, and 0.1°/sec. Although β changes tenfold, the change in $(T_r - T_0)$ is less than 50% in the linear interval of the curve. We conclude that the retention temperature is not very sensitive to changes in β (or t^0 or $1/a$) in the linear, high temperature region. It can be shown to an approximation that T_r increases only as the logarithm of the heating rate in this region. The calculated dependence of T_r on β is shown in Fig. 3 for $\Delta H^0 = 6{,}000$ and 10,000 cal/mole. A rapid increase with β is observed only at very small β values.

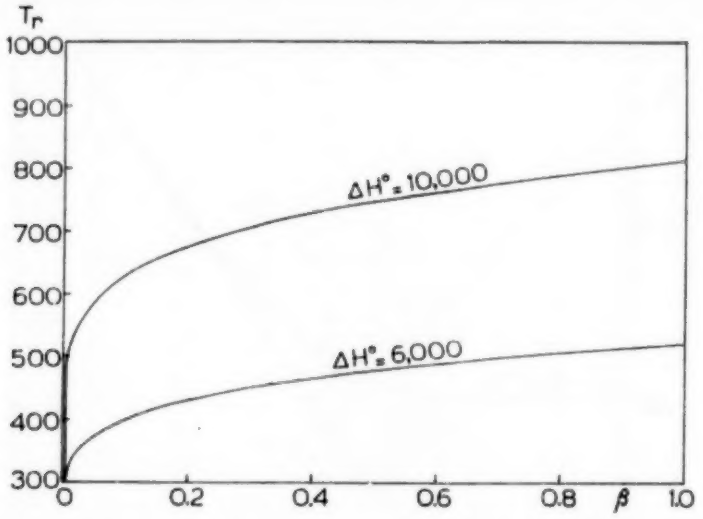

Fig. 3. Retention temperature *versus* heating rate for different enthalpy values.

The retention time, $t_r = (T_r - T_0)/\beta$, in contrast to the retention temperature, decreases rapidly with increasing values of β. This is illustrated in Fig. 4 with ΔH^0 values the same as in Fig. 3.

Mention has been made occasionally of equations that are valid at high temperatures (see eqns. (14) and (18)). In these cases the term containing X_0 becomes negligible compared to the term containing X_r. The range of validity of this approximation is best illustrated by reference to Fig. 5. Retention temperature curves are shown for different initial temperatures. It is seen that the influence of the initial temperature disappears rapidly, and the retention temperature curves all merge together at temperatures slightly greater than T_0. In all cases high temperature approximations may accurately be made within 100° of T_0.

Reference has been made to the retention temperature curve for a homologous

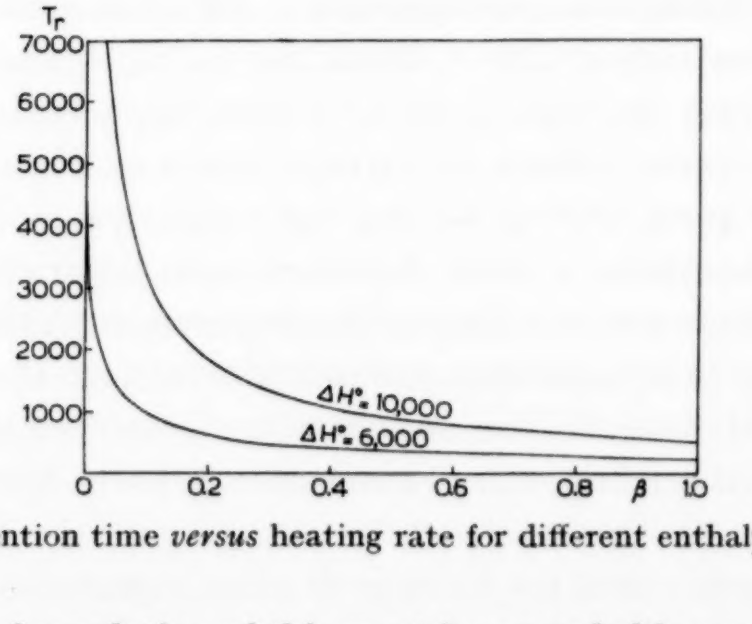

Fig. 4. Retention time *versus* heating rate for different enthalpy values.

series. A quantitative formulation of this must be preceded by an expression relating the thermodynamic terms, ΔH^0 and ΔS^0, to the number of carbon atoms, n. It is a common assumption in chromatographic theory[5] that such terms increase linearly with n. While much evidence supports this assumption, other evidence is conflicting[6]. We will use the assumption here for lack of a more conclusive relationship. The standard enthalpy and entropy changes can consequently be written as $\Delta H^0 = h_0 + nh$ and $\Delta S^0 = s_0 + ns$.

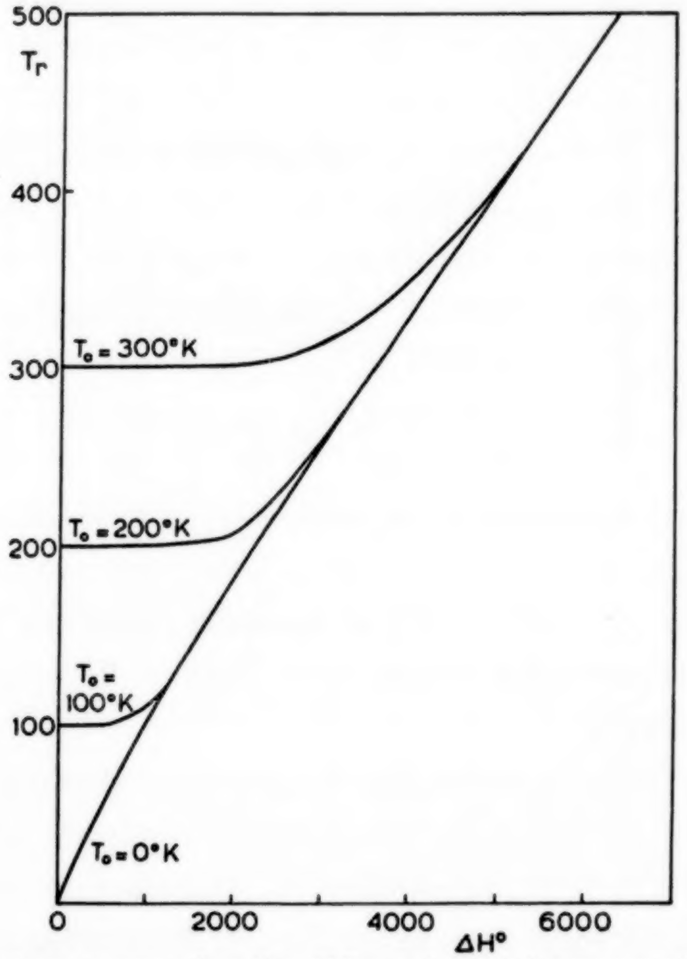

Fig. 5. Retention temperature curves for the different initial temperatures, $T_0 = 0°$ K, 100° K 200° K, 300° K.

In isothermal experiments changes in ΔF^0 are directly measurable in terms of changes in R_F. However it is not important whether the change is primarily in enthalpy or in entropy. With programmed temperature chromatography the relative enthalpy–entropy contributions are important in determining the overall nature of the retention temperature curves. This is illustrated in Fig. 6 where T_r values are calculated from eqn. (13). In both cases $h_0 = 3000$ cal/mole and $h = 750$ cal/mole. However the lower curve reflects an entropy increase, $s = 0.5$ entropy units, for every added carbon atom, whereas for the upper curve $s = 0$. Because of this difference, the lower curve shows a noted departure from linearity. Experimentally obtained plots of T_r *versus* n show a definite bending away from the T_r axis[2]. The experimental cases appear to be somewhere intermediate between the two curves of Fig. 6. It must be cautioned, however, that some bending away from the T_r axis exists with $s = 0$, and additional bending might be caused if h slowly decreases with increasing values of n. Further bending results if the average gas velocity is increased during the run. Thus the term s need not be large at all to explain the experimental results. The problem is in need of further study.

The mathematical equations developed for programmed temperature chromatography are, of course, limited by the assumptions used. Several of the assumptions will be briefly discussed in order to evaluate their importance to the final results.

The average gas velocity in a column is an operating variable that can be held constant or varied during a run. For the development of our specific equations, starting with eqn. (8), we have assumed the gas velocity to remain constant in time. Some equipment, however, operates with a constant mass flux, which means the exit velocity increases linearly with time. Hence the higher boiling solutes would be eluted with a greater average velocity than the lower boiling solutes. This effect is compensated to some extent by the higher inlet pressure (and slower inlet velocity) required to effect a velocity increase and also to overcome the increasing gas viscosity. MARTIN[7], for example, has reported that t^0 changes approximately 15% (rather than 100%) in going from room temperature to 300°C. Furthermore the effect of variations in velocity are reduced in importance by virtue of the fact that the retention temperature varies only as the logarithm of velocity. It is to be emphasized here that if a linear increase in velocity is observed, the modified retention temperature can be calculated with little additional difficulty by substituting $v_0 + \gamma t$ for v in eqn. (7), and using the same type of approximate integration as appears in eqn. (12). A linear velocity increase will lower the retention temperature of the slowly moving species, and will cause the retention temperature curve to bend away from linearity as mentioned earlier.

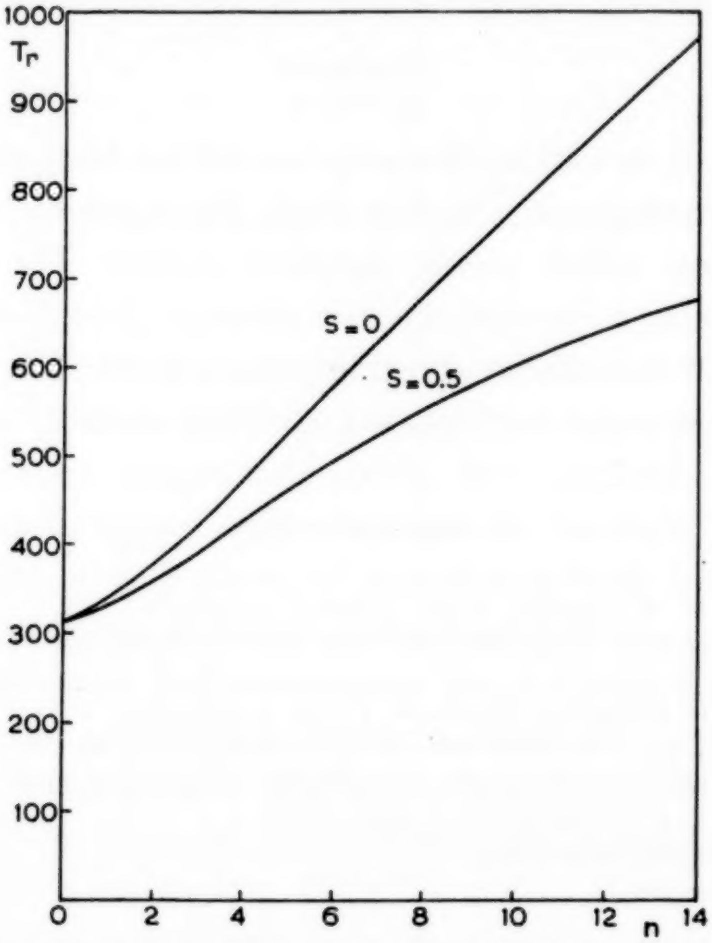

Fig. 6. Dependence of retention temperature on number of carbon atoms with the different entropy increments, $s = 0.5$, $s = 0$.

The assumption regarding R_F, in which eqn. (5) is replaced by eqn. (6), introduces considerable simplicity into the equations. Eqn. (5) can be left intact at the expense of numerically integrating eqn. (7) to obtain t_r.

The use of eqn. (6) for solutes with low ΔH^0 values is obviously wrong, since this approximation can lead to R_F values greater than unity. The error in t_r is, however, only the order of t^0. Errors of the same magnitude will be found for larger ΔH^0 values, but the relative error, except at exceedingly high heating rates, will be negligible.

SUMMARY

A theoretical analysis of retention times and retention temperatures in programmed temperature gas chromatography is described. The analysis is implemented by an approximate integration which yields excellent results. The effects of changes in heating rate, column length, percentage of stationary liquid, etc., are established. A number of examples are calculated showing these effects. The retention temperature curve for a homologous series is discussed, and the various factors influencing this are noted.

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NACHWEIS VON CARBOHYDRASEN-AKTIVITÄT IM HANDELSPAPAIN DURCH PAPIERCHROMATOGRAPHIE

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Das Handelspapain weist eine vielfältige Hydrolasenaktivität auf, wobei neben der hohen proteolytischen Wirksamkeit[1] besonders die esteratischen, lipatischen und pectolytischen Aktivitäten ausgeprägt sind[2]. Die vorliegende Untersuchung soll zeigen, dass das Handelspapain auch eine carbohydratische Wirksamkeit besitzt, worüber in der kürzlich von ESCHRICH[3] veröffentlichten Untersuchung über den enzymatischen Kalloseabbau mit Papain hingewiesen wird.

Der Nachweis sowie die Differenzierung der Carbohydrasen-Aktivitäten wird durch die papierchromatographische Identifizierung der Spaltprodukte der verschiedenen Kohlenhydratsubstrate geführt[4]. Die Enzymwirkung durch papierchromatographische Identifizierung ihrer Reaktionsprodukte zu charakterisieren, ist besonders dann die Methode der Wahl, wenn es darum geht, ein komplexes Enzymgemisch zu differenzieren oder ein gereinigtes Enzympräparat auf seine Spezifität hin zu untersuchen[5-10].

METHODIK

Darstellung des Papain-Acetontrockenpräparates

Die gut verrührte Suspension von 5 g Papainpulver in 50 ml dest. Wasser wird 10 min bei 4000 U/min zentrifugiert und der Wasserauszug wird mit dem gleichen Volumen Aceton bei etwa + 3° versetzt; der gebildete Niederschlag wird abzentrifugiert und im Vakuum bei — 4° getrocknet. Das so gewonnene Acetontrockenpräparat (AcT) macht etwa 40 % des eingewogenen Papainpulvers aus.

Enzymversuche

Zur Herstellung der Enzymlösung löst man 0.5 g AcT in 5 ml Phosphatpuffer pH 6.8 oder Acetatpuffer pH 5.0 und zentrifugiert den unlöslichen Rückstand ab. Die Versuchsansätze bestanden aus jeweils 0.5 ml 5 % wässriger Substratlösung und 0.5 ml Enzymlösung, sowie als Leerversuche aus 0.5 ml Enzymlösung und 0.5 ml Wasser bzw. zur Feststellung einer eventuellen Eigenspaltung aus 0.5 ml Pufferlösung und 0.5 ml wässriger Substratlösung. Die Ansätze wurden zur Vermeidung einer Infektion durch Mikroorganismen mit Toluol überschichtet. Als Substrate wurden an Polysacchariden: Stärke, Glykogen und Dextran, an Trisacchariden: Melecitose und Raffinose, und an Disacchariden: Maltose, Cellobiose, Trehalose, Saccharose, Melibiose und Lactose verwendet. Als Inhibitor-Substanz verwendeten wir pro Versuchsansatz 500 γ Monojodessigsäure. Die Versuchsansätze wurden 24 h bei 37° inkubiert.

Davon wurde zu den Zeiten t_0 und t_{24} eine Probe von 0.01 ml papierchromatographisch mittels der aufsteigenden Technik auf Spaltprodukte der Kohlenhydrate untersucht. Als Fliessmittel diente das Gemisch *n*-Butanol–Eisessig–Wasser (4:1:5)[11]. Die Aldosen wurden durch ihre braune Färbung mit Anilin–Phthalsäure[12] und die Ketosen durch ihre grüne Färbung mit Harnstoffe-HCl[13] nachgewiesen. Die Identifizierung der lokalisierten Zucker geschah auf Grund ihrer R_F-Werte sowie mit Hilfe mitchromatographierter Testsubstanzen.

ERGEBNISSE

Tabelle I gibt einen qualitativen Überblick über die enzymatische Wirksamkeit des Papainacetonpräparates unter verschiedenen Bedingungen gegenüber einer Reihe von Kohlenhydraten als Substrate.

TABELLE I

Substrat	*Phosphatpuffer pH 6.8*	*Acetatpuffer pH 5.0*	*Phosphatpuffer pH 6.8 + Monojodessigsäure*
Stärke	++	—	—
Glykogen	++	—	—
Dextran	—	—	—
Melecitose	—	—	—
Raffinose	+++	+++	++
Saccharose	+++	++++	++
Maltose	++	+	—
Trehalose	++	+	++
Cellobiose	+	+	—
Lactose	+	+	—
Melibiose	+	+	+

— Keine Aktivität, + schwache Aktivität, ++ mittlere Aktivität, +++ starke Aktivität, ++++ sehr starke Aktivität.

Von den Polysacchariden werden Stärke und Glykogen, deren Glucosereste durch 1,4-Bindungen miteinander verknüpft sind, bei pH 6.8 bis zu Glucose aufgespalten (Fig. 1). Dagegen lässt sich bei pH 5.0 in Acetatpuffer eine amylolytische Aktivität nicht mehr beobachten. Durch Zusatz von Monojodessigsäure wird auch bei pH 6.8 die Amylase-Aktivität völlig aufgehoben. Ob es sich um eine α- oder β-Amylase handelt, kann natürlich aus den vorliegenden Versuchen nicht abgeleitet werden. Bisher wurde nur in frischem Papaya-Latex eine deutlich feststellbare Amylase-Aktivität[14] nachgewiesen. Dextran, das hauptsächlich aus α-1,6-glucosidisch gebundenen Glucoseresten besteht, wird nicht angegriffen.

Das Papainpulver weist eine vielfältige enzymatische Spaltungsaktivität gegenüber den verschiedenen Oligosacchariden auf, was auf das Vorkommen ver-

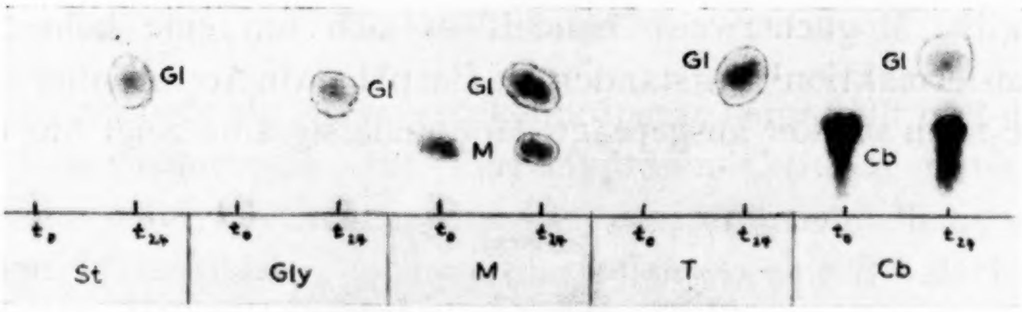


Fig. 1. Chromatogramm der Versuchsansätze in Phosphatpuffer. t_0 = Beginn der Inkubation; t_{24} = Inkubation nach 24 h. Fliessmittel: *n*-Butanol–Eisessig–Wasser (4:1:5); Entwickler: Anilin–Phthalsäure. St = Stärke; Gly = Glykogen; M = Maltose; T = Trehalose; Cb = Cellobiose; Gl = Glucose.

schiedener Carbohydrasen schliessen lässt. Wie aus Fig. 1 ersichtlich ist, werden Cellobiose, Maltose und Trehalose, die sich sterisch bzw. bindungsmässig unterscheiden, bei pH 6.8 zu Glucose aufgespalten, was auf das Vorkommen einer β-Glucosidase sowie mindestens einer α-Glucosidase hinweist. Die Trehalose selbst wird auf dem Chromatogramm infolge Blockierung der reduzierenden Gruppen durch Anilin–Phthalsäure nicht angefärbt. Bei pH 5.0 in Acetatpuffer kann man auf dem Chromatogramm bei der Maltosespaltung nur noch einen schwachen Glucosefleck und bei der Trehalosespaltung nur noch Spuren von Glucose feststellen. Hingegen hemmt Monojodessigsäure bei pH 6.8 in Phosphatpuffer die Maltosespaltung fast vollständig und die Trehalosespaltung bleibt unbeeinflusst. Dieses unterschiedliche Verhalten des Enzympräparates gegenüber den beiden α-Glucosiden Trehalose (α-1,1-Glucosido-glucosid) und Maltose (α-1,4-Glucosido-glucosid) deutet auf das Vorliegen von zwei α-Glucosidasen hin, die für die Spaltung der α-1,1- sowie die der α-1,4-glucosidischen Bindung spezifisch eingestellt sind. Die beiden Galactoside Lactose (β-4-Galactosido-glucose) und Melibiose (α-6-Galactosido-glucose) werden zu Glucose und Galactose abgebaut (siehe Fig. 2), wobei durch Monojodessigsäure der enzymatische Lactoseabbau vollständig und der Melibioseabbau teilweise gehemmt werden (Tabelle I). Somit kann man auf die Existenz einer β-Galactosidase und einer α-Galactosidase schliessen.

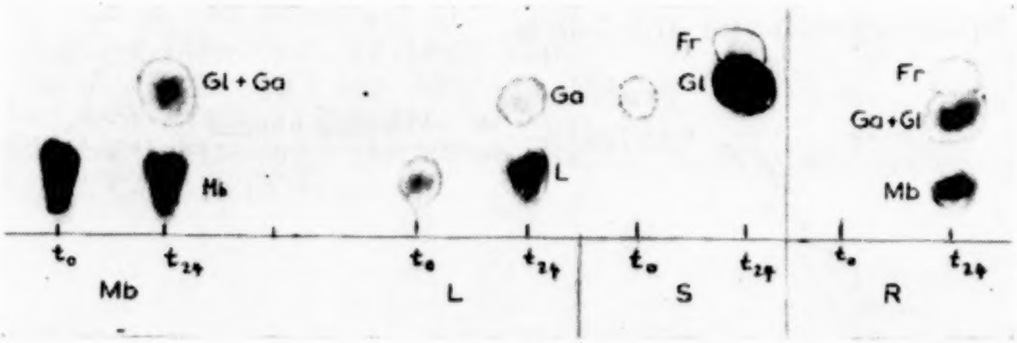


Fig. 2. Chromatogramm der Versuchsansätze in Phosphatpuffer. Fliessmittel: *n*-Butanol–Eisessig–Wasser (4:1:5). Entwickler: Anilin–Phthalsäure. Mb = Melibiose; L = Lactose; S = Saccharose; R = Raffinose; Ga = Galactose; Fr = Fructose.

Saccharose wird sehr stark in ihre Komponenten gespalten, wie die scharf ausgeprägten Glucose- und Fructoseflecken auf dem Chromatogramm beweisen (Fig. 2 und 3). In Fig. 3 ist ausserdem ein Fleck (O) sichtbar, der sich durch Harnstoff-HCl

Anfärbung ergibt. Möglicherweise handelt es sich um eine höhere Ketose, die durch eine Transferreaktion[15] entstanden ist. Bei pH 5.0 in Acetatpuffer ist die Spaltung der Saccharose noch stärker ausgeprägt. Monojodessigsäure zeigt nur eine schwache Hemmwirkung auf den Saccharose-Abbau. Es ist also anzunehmen, dass die Saccharose hauptsächlich von einer β-h-Fructosidase angegriffen wird. Nur in geringerem Umfang dürfte die α-Glucosidase, die für die Maltosespaltung charakteristisch ist, dafür in Frage kommen, denn bei pH 5.0 sowie in Gegenwart von Monojodessigsäure zeigt sie das gegenteilige Verhalten wie die Fructosidase. Ebenso zeigt das Enzympräparat das gleiche Verhalten gegenüber Raffinose, wie gegenüber Saccharose (Fig. 2 und 3). Die β-h-Fructosidase spaltet aus dem Trisaccharid den Fructoseteil ab und die entstandene Melibiose, die sich durch ihre braune Anfärbung mit Anilin-Phthalsäure auf dem Chromatogramm (Fig. 2) abhebt, wird ihrerseits durch die α-Galactosidase, wenn auch in schwächerem Umfang, in Glucose und Galactose hydrolysiert. Der bei t_{24} (Fig. 3) entstandene Saccharosefleck beweist, dass das Raffinosemolekül auch unter Aufspaltung der α-1,4-galactosidischen Bindung durch die α-Galactosidase angegriffen wird. Somit lässt sich für Papain gegenüber Raffinose folgendes enzymatische Spaltungsschema aufstellen:

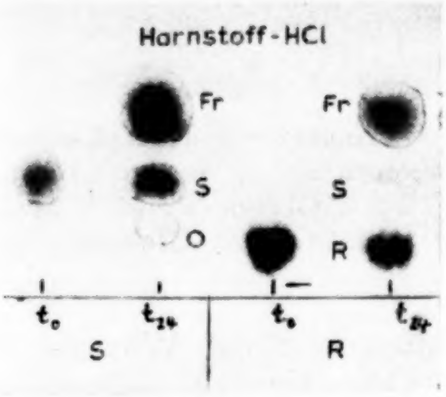
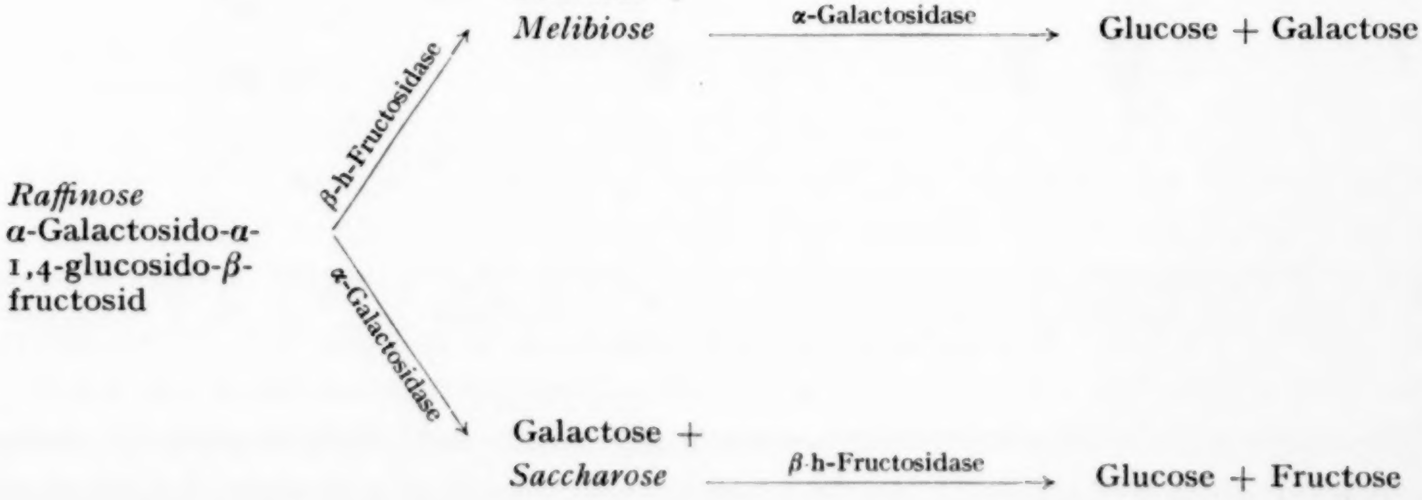

Harnstoff-HCl
Fr
Fr
S
S
O
R
t_0
t_{24}
t_0
t_{24}
S
R

Fig. 3. Chromatogramm der Versuchsansätze in Phosphatpuffer. Fliessmittel: *n*-Butanol–Eisessig–Wasser (4:1:5); Entwickler: Harnstoff–HCl. O = Oligosaccharid.


Fructose +
Melibiose
α-Galactosidase
Glucose + Galactose
β-h-Fructosidase
Raffinose
α-Galactosido-α-1,4-glucosido-β-fructosid
α-Galactosidase
Galactose +
Saccharose
β-h-Fructosidase
Glucose + Fructose

Das Trisaccharid Melecitose, an deren Fructoseteil noch ein Glucoserest hängt, wird von dem Acetonpräparat fermentativ nicht angegriffen. Es scheint demnach im Papain ausschliesslich die β-h-Fructosidase vorzuliegen, die nur Oligosaccharide hydrolysiert, deren Fructoseteil nicht substituiert ist[16].

ZUSAMMENFASSUNG

Aus Handelspapain wurde ein Acetontrockenpräparat hergestellt und mittels papierchromatographischer Methodik auf Carbohydrasen-Aktivität untersucht. Dabei konnten neben einer amylolytischen Aktivität zwei unterschiedliche α-Glucosidase-Wirkungen, eine β-Glucosidase-, eine α-Galactosidase-, eine β-Galactosidase- sowie eine β-h-Fructosidase-Wirkung nachgewiesen werden.

SUMMARY

The carbohydrase activity of a dried acetone preparation obtained from commercial papain was investigated by paper chromatography. Besides amylase activity, two different α-glucosidases, a β-glucosidase, an α-galactosidase, a β-galactosidase and a β-h-fructosidase were found.

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THIN LAYER CHROMATOGRAPHY OF WEAKLY POLAR STEROIDS*

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INTRODUCTION

The analytical separation of slightly polar steroids has been realized by paper partition chromatography on impregnated[1,2] or acetylated paper[3]. These methods have, however, some drawbacks, such as the long time of development. For the *preparative* separation of weakly polar steroids adsorption chromatography is superior to partition chromatography in simplicity and speed[4], but rapid *analysis* needs an "open column", a counterpart of the paper strip and sheet. This need has been filled by the adsorbent-coated glass strip (chromatostrip[5]) and plate (chromatoplate[6]), which allow micro-adsorption chromatography on a flat surface (thin layer chromatography[7]). A review of this method has been published[8]; most applications are in the field of essential oils and terpenes[5-10], but some other lipophilic compounds have also been separated[8-11]. In the steroid field, thin layer chromatography of bile acids has been mentioned but without experimental details[10].

In order to investigate the possibilities of this technique in the steroid domain we tried to separate cholesterol, cholesterol esters, cholestenediols and some steroid hydroxyketones. After preliminary experiments with many solvent mixtures we succeeded in separating these compounds (Figs. 1, 2).

For our investigations into the oxidative degradation of the cholesterol side chain[12], it was necessary to carry out the analytical separation of the neutral fractions. When preparing androstenolone acetate (3β-acetoxy-5-androsten-17-one) by oxidation of cholesteryl acetate dibromide with chromic oxide and debromination with zinc (*cf.*[12] and references cited there) many other degradation compounds are formed[13]. Various neutral by-products have been isolated by adsorption chromatography[14], but an analytical separation has not been described.

However, a paper chromatographic separation of these fractions is possible[1,2]. A complete separation was realized by two-dimensional paper chromatography, which took several days. However, by using thin layer chromatography we succeeded in resolving the neutral oxidation fractions within 90 min into more than 20 compounds (Fig. 3). Some of these could be identified with certainty. Apart from androstenolone acetate and unchanged cholesteryl acetate we found three known[15] constituents:

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[*] Steroids. Part II. For Part I see ref.[12].

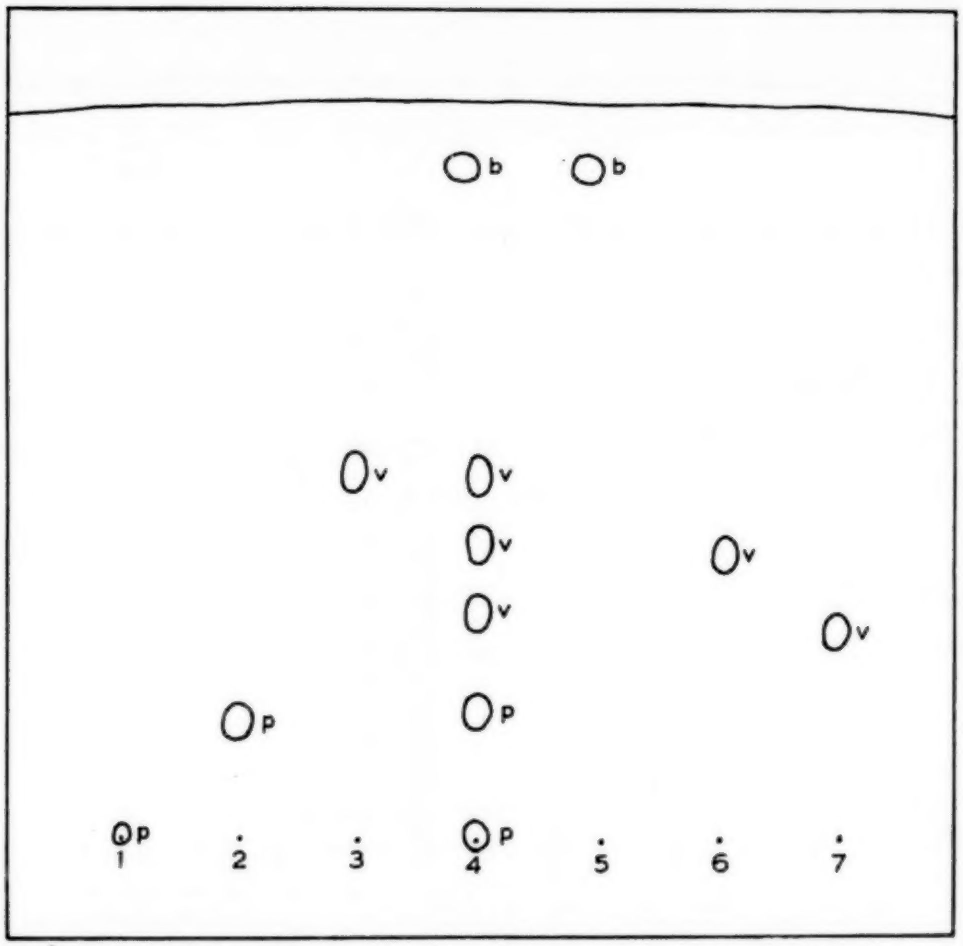

Fig. 1. Solvent: CB1*. Time of development: 85 min. 1 = 10 μg cholesterol; 2 = 10 μg cholesteryl acetate; 3 = 10 μg cholesteryl palmitate; 4 = mixture of 1, 2, 3, 5, 6 and 7; 5 = 10 μg cholesteryl chloride; 6 = 10 μg cholesteryl benzoate; 7 = 10 μg cholesteryl monochloroacetate. Colours are indicated*.

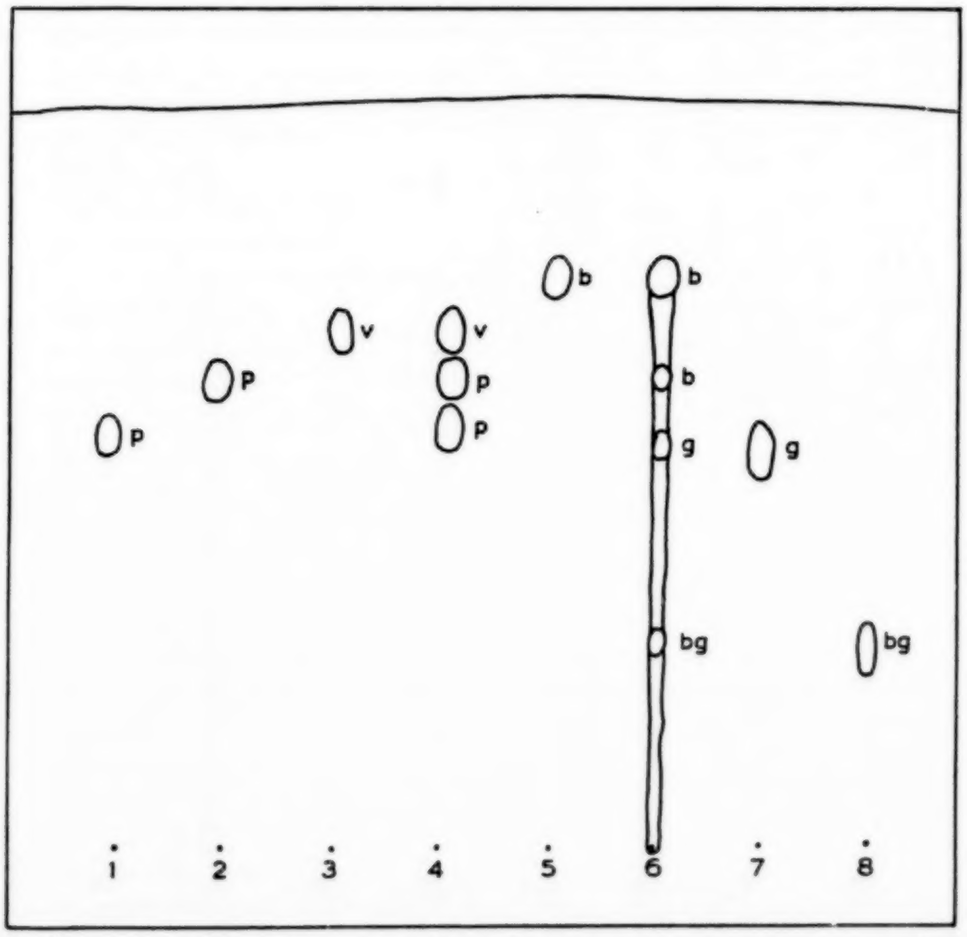

Fig. 2. Solvent: BE2*. Time of development: 80 min. 1 = 5 μg 5-androsten-3β-ol-17-one; 2 = 5 μg 5-pregnen-3β-ol-20-one; 3 = 5 μg 26-nor-5-cholesten-3β-ol-25-one; 4 = mixture of 1, 2 and 3; 5 = 10 μg cholesterol; 6 = 30 μg residue of cholesterol mother liquors; 7 = 10 μg 25-hydroxycholesterol; 8 = 5 μg 7α-hydroxycholesterol. Colours are indicated*.

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* For abbreviations see Table II.

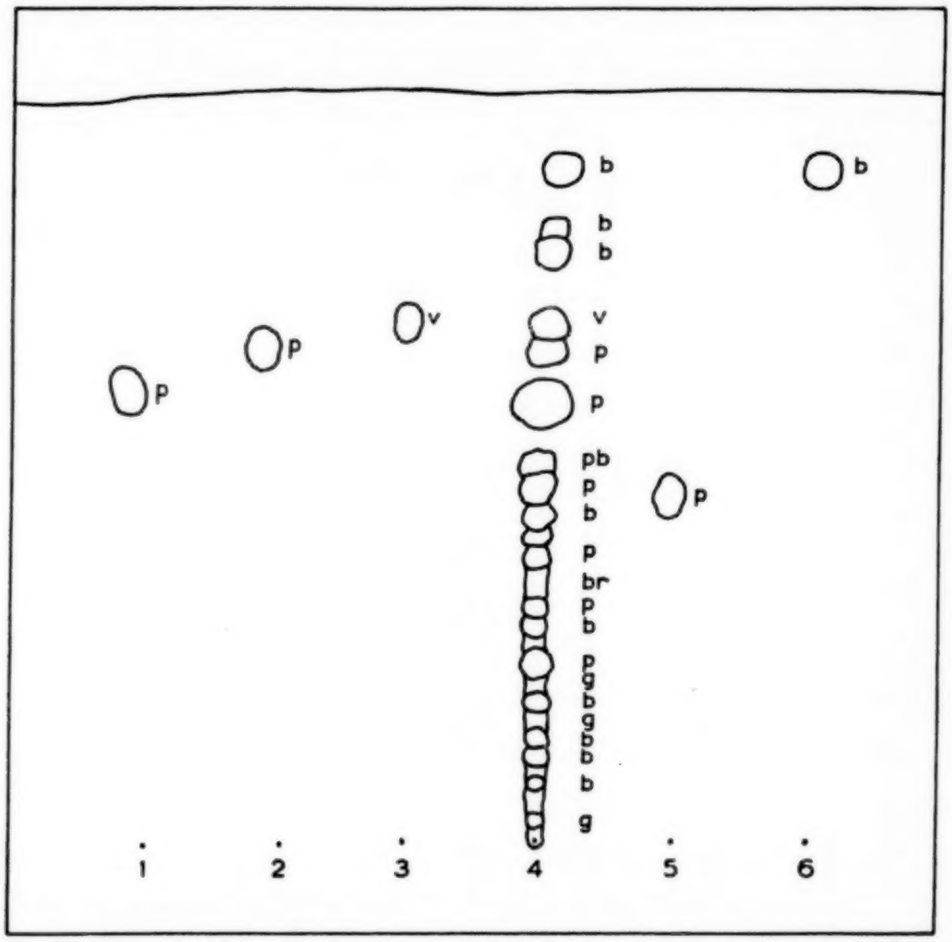

Fig. 3. Solvent: TE2*. Time of development: 45 min. 1 = 10 µg 3β-acetoxy-5-androsten-17-one; 2 = 10 µg 3β-acetoxy-5-pregnen-20-one; 3 = 10 µg 3β-acetoxy-26-nor-5-cholesten-25-one; 4 = 50 µg neutral fraction of a cholesteryl acetate dibromide oxidation: this fraction appeared to contain 22 ± 2% of 1, 4 ± 1% of 2, 3 ± 1% of 3, 3 ± 1% of 5 and 12 ± 2% of 6 by semi-quantitative determination; 5 = 10 µg 3β-acetoxy-5-cholen-(24,20)-olide; 6 = 10 µg cholesteryl acetate. Colours are indicated*.

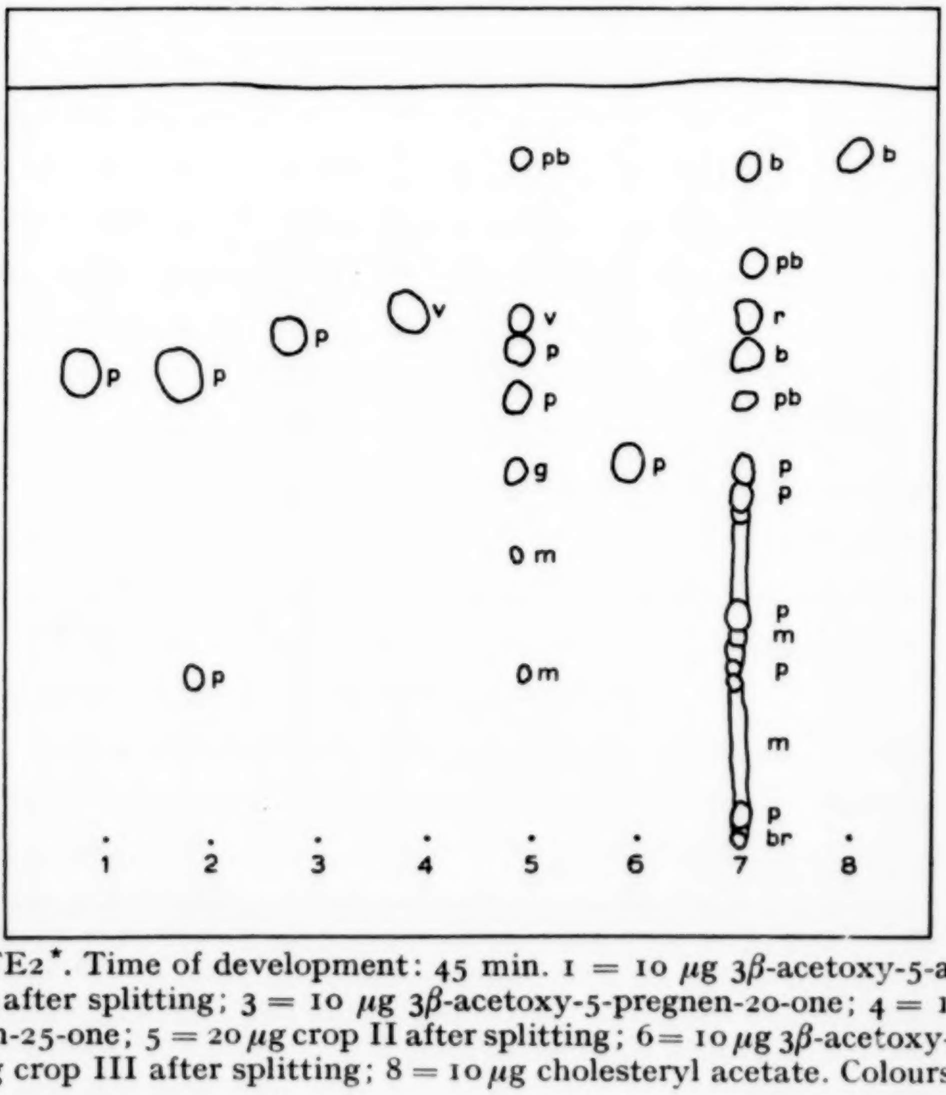

Fig. 4. Solvent: TE2*. Time of development: 45 min. 1 = 10 µg 3β-acetoxy-5-androsten-17-one; 2 = 20 µg crop I after splitting; 3 = 10 µg 3β-acetoxy-5-pregnen-20-one; 4 = 10 µg 3β-acetoxy-26-nor-5-cholesten-25-one; 5 = 20 µg crop II after splitting; 6 = 10 µg 3β-acetoxy-5-cholen-(24,20)-olide; 7 = 20 µg crop III after splitting; 8 = 10 µg cholesteryl acetate. Colours are indicated*.

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* For abbreviations see Table II.

pregnenolone acetate (3β-acetoxy-5-pregnen-20-one), norcholestenolone acetate (3β-acetoxy-26-nor-5-cholesten-20-one) and 3β-acetoxy-5-cholen-(24,20)-olide (*cf.*[16] and references cited there). The identification of the other substances is under investigation.

In all cases we detected the spots with the well-known antimony trichloride reagent[1,10]. We realized a semi-quantitative determination of the more important constituents of the neutral fractions by visual comparison of the spots with those of known quantities of these compounds[2,17]. An example is given in Fig. 3. By treatment with semicarbazide, the neutral fractions were divided into three crops[14], which were afterwards split[18]. The distribution of the different compounds over these crops is shown in the chromatogram of Fig. 4.

Finally we mention another application of thin layer chromatography in the sterol field: the separation of contaminations of cholesterol. The mother liquors of

TABLE I

APPROXIMATE R_F VALUES OF CHOLESTERYL ACETATE (CA), 3β-ACETOXY-5-PREGNEN-20-ONE (PA) AND 3β-ACETOXY-5-ANDROSTEN-17-ONE (AA) IN VARIOUS SOLVENTS

Solvent	*CA*	*PA*	*AA*
Hexane	0.03	0	0
Heptane	0.03	0	0
Cyclohexane	0.04	0	0
Methylcyclohexane	0.04	0	0
Benzene	0.70	0.14	0.07
Toluene	0.73	0.33	0.28
Ethylbenzene	0.36	0.04	0.02
Dichloromethane*	0.88	0.49	0.32
Chloroform*	0.88	0.63	0.51
Tetrachloromethane	0.17	0	0
1,2-Dichloroethane	0.85	0.40	0.24
1,1,2,2-Tetrachloroethane	0.76	0.26	0.14
Chlorobenzene	0.42	0.04	0.02
o-Dichlorobenzene	0.28	0	0
Nitromethane	0.97	0.84	0.78
Nitrobenzene	0.90	0.38	0.28
Methanol	1	1	1
Ethanol	1	1	1
2-Propanol	1	1	1
1,2-Ethanediol	1	1	1
2-Phenoxyethanol	1	1	1
Diethyl ether	1	1	1
Diisopropyl ether*	0.95	0.81	0.75
Di-*n*-butyl ether	0.80	0.33	0.21
Anisole	0.90	0.47	0.35
Phenetole	0.85	0.47	0.35
Dioxan	1	1	1
Acetone	1	1	1
Acetophenone	1	1	1
Acetic acid	1	1	1
Ethyl acetate	1	1	1
Acetonitrile	1	1	1
Pyridine	1	1	1

* Solvent volatility causes changing R_F values.

cholesterol appeared to contain three other compounds, including 7α-hydroxycholesterol[19] and 25-hydroxycholesterol[20] or other substances with the same R_F values (Fig. 2).

An outline of the R_F values, found in the different experiments, is given in Tables I and II. In all these cases we succeeded in finding one or more solvent mixtures that gave rapid and good separations.

EXPERIMENTAL

General conditions

Smooth glass plates (thickness 0.37 cm), oblong (5 × 20 cm) and square (20 × 20 cm) were used. They were covered with a thin, even layer of Silica Gel G* (a standardized mixture of silicic acid and gypsum) by spreading a well-stirred mixture of 5 parts of Silica Gel G and 9 parts of distilled water[10] with a "thin layer applier"**. This layer was activated by heating for 30 min at 105°; the chromatostrips and chromatoplates were kept in a closed jar with a drying agent.

The starting points were marked[7,8] on the strips and plates, in most cases at 2 cm from the base line with a distance of 2 cm in between. Then 0.1–150 μg of substance, dissolved in 1–10 μl of a volatile solvent, was put on the marked points with a sharpened 10 μl micropipet***. The solvent evaporated rapidly.

To determine a certain fraction on a chromatoplate semi-quantitatively, we applied alternately a fixed amount of that fraction and regularly increasing quantities of one or more of the constituents[2,17].

We applied the ascending technique[5]. The chromatostrips were placed in glass cylinders, closed by a cork, ground glass plate or stopper. Their internal height was 22–24 cm, internal diameter 5.5 cm. The strips were mainly used for preliminary experiments. The chromatoplates were placed in glass jars (height and length 21–24 cm, width 10–20 cm), covered with ground glass plates and, if necessary, fitted with glass racks to keep the chromatoplates upright.

For development, the cylinders and jars were filled with a quantity of solvent mixture to wet the strips and plates to a height of *ca.* 0.5 cm. Shortly before the solvent front was about to reach the top, the development was interrupted. The time of development depends on the temperature, the physical properties of the solvent and the possibility of evaporation; development took mostly 40–100 min.

After development the strips and plates were taken out of the cylinders or jars and their front line was marked. After thoroughly drying at 105° they were sprayed with a saturated solution of antimony trichloride in chloroform[1,10] and heated for 5 min at 105°. The colours of the spots varied; most spots were purple, but some were blue, green, brown or rose. The fading of the spots could be slowed down by covering the strips and plates with ordinary glass plates.

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TABLE II

APPROXIMATE R_F VALUES IN SOME SOLVENTS AND SOLVENT MIXTURES

Substance	*Solvent**													*Colours***				
	B	*BE1*	*BE2*	*C*	*CB1*	*CB2*	*CE1*	*CE2*	*D*	*H*	*T*	*TE1*	*TE2*	*B*	*BE2*	*D*	*T*	*TE2*
Cholesterol	0.10	0.39	0.72	0	0.01	0.05	0.14	0.25	0.25	0	0.16	0.33	0.40	p	v	v	v	p
Cholesteryl acetate	0.70	0.94	0.96	0.03	0.17	0.50	0.79	0.86	0.85	0.03	0.73	0.92	0.92	v	v	v	b	p
Cholesteryl monochloroacetate	0.87	0.96	0.97	0.04	0.34	0.73	0.85	0.91	0.94	0.04	0.89	0.95	0.97	v	v	v	v	v
Cholesteryl palmitate	0.93	0.97	1	0.08	0.53	0.87	0.95	0.97	0.96	0.06	0.98	0.98	0.99	v	b	v	b	v
Cholesteryl oleate	0.94	0.97	1	0.05	0.47	0.89	0.94	0.97	0.96	0.04	0.98	0.98	0.98	v	v	r	v	p
Cholesteryl benzoate	0.91	0.97	1	0.06	0.44	0.80	0.93	0.95	0.95	0.05	0.94	0.97	0.99	v	v	v	v	p
Cholesteryl chloride	0.98	0.98	1	0.75	0.93	0.95	0.96	0.98	0.96	0.75	0.99	1	1	v	v	v	v	v
5-Cholestene-3β,7α-diol	0	0.03	0.21	0	0	0	0	0.01	0.01	0	0.01	0.03	0.05	bg	bg	bg	bg	bg
5-Cholestene-3β,25-diol	0.01	0.10	0.41	0	0	0	0.01	0.05	0.03	0	0.02	0.10	0.16	br	g	g	gb	p
5-Androsten-3β-ol-17-one	0.02	0.17	0.51	0	0	0	0.01	0.05	0.06	0	0.07	0.15	0.21	p	p	p	p	p
5-Pregnen-3β-ol-20-one	0.03	0.21	0.57	0	0	0	0.04	0.09	0.08	0	0.08	0.19	0.28	p	p	p	p	p
26-Nor-5-cholesten-3β-ol-25-one	0.04	0.24	0.63	0	0	0	0.05	0.10	0.09	0	0.09	0.23	0.34	p	v	p	b	p
3β-Acetoxy-5-androsten-17-one	0.07	0.60	0.86	0	0	0.02	0.16	0.33	0.24	0	0.28	0.51	0.60	p	v	p	p	p
3β-Acetoxy-5-pregnen-20-one	0.14	0.71	0.89	0	0	0.03	0.23	0.43	0.40	0	0.33	0.59	0.67	p	v	p	p	p
3β-Acetoxy-26-nor-5-cholesten-25-one	0.19	0.74	0.91	0	0	0.04	0.29	0.47	0.45	0	0.36	0.60	0.71	r	v	r	b	v
3β-Acetoxy-5-cholesten-7-one	0.69	0.93	0.96	0.04	0.16	0.49	0.78	0.88	0.87	0.03	0.70	0.88	0.91	r	b	r	r	b
3β-Acetoxy-5-cholen-(24,20)-olide	0.06	0.44	0.80	0	0	0.01	0.05	0.14	0.28	0	0.23	0.40	0.47	p	v	p	b	p

* Abbreviations: B = benzene; BE1 = benzene–ethyl acetate, 9:1 (v/v); BE2 = benzene–ethyl acetate, 2:1 (v/v); C = cyclohexane; CB1 = cyclohexane–benzene, 4:1 (v/v); CB2 = cyclohexane–benzene, 1:1 (v/v); CE1 = cyclohexane–ethyl acetate, 19:1 (v/v); CE2 = cyclohexane–ethyl acetate, 9:1 (v/v); D = 1,2-dichloroethane; H = hexane; T = toluene; TE1 = toluene–ethyl acetate, 19:1 (v/v); TE2 = toluene–ethyl acetate, 9:1 (v/v).

** Colours of the spots with the antimony trichloride reagent; abbreviations: b = blue; bg = bluish green; br = brown; g = grey; gb = greyish blue; m = mauve; p = purple; pb = pale blue; r = rose; v = violet.

For inspection, the coloured chromatostrips and chromatoplates were put on an illuminated glass plate; if necessary, semi-quantitative estimation by visual comparison[2,17] was carried out immediately after colouring and the plates were copied or photographed[10]. Colour slides provide accurate records of the chromatograms.

Special experiments

Cholesterol and its esters came from our own factory; the steroid ketones and lactone were commercial products. 7α-Hydroxycholesterol was prepared from cholesteryl hydrogen phthalate[19] and 25-hydroxycholesterol was isolated from aged cholesterol[20].

For the analysis of contaminations, cholesterol was crystallized from methanol; after three crops had been separated, the final mother liquors were evaporated and the residue chromatographed.

The oxidations of cholesteryl acetate dibromide were carried out in the usual way[12]. The neutral fractions were divided into three crops by treatment with semicarbazide acetate[14]. Before chromatography, the crops were split[18].

The solvent mixtures used are mentioned in Tables I and II.

ACKNOWLEDGEMENTS

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SUMMARY

Slightly polar steroids, for example cholesterol esters and 3β-acetoxy-steroid ketones, can be separated by thin layer chromatography in a short time. Neutral fractions of oxidation products from side chain degradations of cholesteryl acetate were separated into more than twenty constituents and some of these could be determinated semiquantitatively. This method gives rapid and good separations.

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A PRACTICAL EXPERIMENTAL METHOD FOR CONVERTING ELECTROPHORETIC MOBILITIES IN PAPER-STABILIZED MEDIA TO FREE SOLUTION VALUES

THE ISOELECTRIC POINTS OF HUMAN SERUM LIPOPROTEINS*

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INTRODUCTION

In a review of the factors which influence the ionographic mobility of a migrant, McDONALD and co-workers[1] presented a view of the mechanics of electromigration in buffer systems stabilized with paper which they found to be consistent with the experimental facts as they were known at the time. They referred to this view as the "barrier theory". According to the present version of the theory the paper fibers act as barriers in the path of migration, their effect differing for migrants of different molecular volume, shape, charge, charge distribution, etc. The effect can be pictured as being due to the fact that the molecules of the migrant suffer mechanical, physical and chemical interaction with the paper fibers and are therefore slowed down. While the migrant is undergoing its forward movement, however, it is doing so essentially in the buffer solution trapped in the interstices between the paper fibers, and the actuating potential gradient, in V/cm, is given by the potential impressed across the paper's length divided by the length of the paper.

On classical grounds it has been pointed out that in electromigration in stabilized electrolytes, the mobility of positively charged ions might be expected to increase while that of negatively charged ions might be decreased, due to the phenomenon of electro-osmosis[2]. In the case of electrolytes stabilized with paper, this conclusion is based on the observation that the paper surface generally appears to bear a negative charge, while the aqueous solution in contact with it acts as if it were positively charged. Consequently, the resultant mobility vector of the positive ions would be expected to be the sum of the simple electromigration mobility and the electro-osmotic mobility of the solution in which they are being buoyed along. Negative ions, on the other hand, may be pictured as breasting the electro-osmotic tide. The resultant mobility vector in the latter case would then be the vector difference between the simple electromigration mobility and the electro-osmotic mobility of the liquid.

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KUNKEL AND TISELIUS[3,4] have considered the electromigration path of a migrant as a sort of meandering tunnel or wormhole through the paper. They made the assumption that, for a given paper, all migrants follow along similar paths and that, since the path is much longer than the actual length of the paper, the potential gradient actuating the migrating particle will be less than the value given by simply dividing the potential difference impressed across the paper strips by their length. On this basis, then, and after applying a correction factor for electro-osmosis,using dextran as an indicator of electro-osmotic movement, they applied a second correction factor for "added migration path length", which appears to bring the mobility of the migrant up to that observed in free solution electrophoresis. They state that this latter correction factor, for a given type of paper, can be used for mobility calculation under widely varying conditions. In other words, the correction factor for "added migration path length" is a function principally of the paper and not of the migrant.

MCDONALD AND SPITZER[5] have pointed out that if the view of KUNKEL AND TISELIUS is correct, it should be found that if the mobilities of different migrants are determined on several varieties of paper, the percentage change in mobility for, say, migrant X from, say, paper A to paper B to paper C should be the same as for a different migrant, Y. They found experimentally that this situation does not obtain. While the mobility of aspartic acid is practically the same on three different papers, the mobility of polyvinylpyrrolidone varies approximately 50% for two different papers. These experimental data can be interpreted to mean that different migrants follow quite different migration paths and that therefore, no single correction for so-called "added migration path length" can be applied for different migrants, even when the same type of paper is used to stabilize the electrolyte.

It would follow then, that in the determination of free solution mobilities by the ionographic technique, not only must the phenomenon of electro-osmosis be considered but the barrier effect must also be considered. The vector components making up the barrier effect comprise any physical or chemical forces, except for electro-osmosis, that would tend to hinder the free movement of a migrating moiety. The barrier effect exists not only for the migrant but also for the electro-osmotic indicator. Various essentially uncharged materials have been used as electro-osmotic indicators. Some of these include dextran, starch, amylose, sucrose[3], creatinine[6], xylose and glucose[7], *o*-nitroaniline and *s*-trinitrobenzene[8], tetramethyl glucose[9], N-β-hydroxyethyl-2,4-dinitroaniline[10], formaldehyde, hydrogen peroxide, thioacetamide, allylthiourea, hydroquinone and radioactive sucrose[11], and caffeine[12]. The technique employed in order to determine the electrophoretic mobility a particular substance would have in free solution electrophoresis has essentially been to determine the electro-osmotic mobility of the indicator and to add or subtract this mobility to or from the "raw mobility" of the negative or positive migrant, respectively. Although KUNKEL, TAYLOR AND DU VIGNEAUD[13], in determining the isoelectric point of oxytocin, did determine the electro-osmotic mobility of their indicator, dextran, at several pH values, workers generally have not taken into account the fact that the barrier effect might be different for the electro-osmotic indicator and the particular migrant under study. In the determination of ionographic isoelectric points, another problem enters the picture, namely the possibility that the barrier effect for a particular migrant might be different on different sides of its isoelectric point.

The present-day need for determination of free solution mobilities from ionographic values stems less from a need to compare the latter values with the former than was the case during the years required for the ionographic technique to grow to full maturity and acceptance. A need for free solution mobilities still exists, however, in connection with the determination of such quantities as isoelectric points, and when attempts are made to determine physical chemical properties of migrants from their mobility behavior in stabilized media. When only micro amounts of a migrant are available or when it has a low molecular weight, *e.g.*, amino acid, inorganic ion, etc., the classical free solution technique cannot be applied. It is in such instances that the determination of free solution mobilities from ionographic values is the only way at the present time to obtain the data in question. The same situation holds when electrophoretic mobilities are to be determined at several different temperatures. Some of the methods whereby free solution mobilities may be determined from ionographic values have been outlined by McDONALD[1] and two of the more theoretically-based treatments have been critically examined by MACWILLIAM[14]. An interesting recent addition to the literature on the subject is the report by EDWARD[15] on the "obstructive factor" in paper electrophoresis and an article on the calculation of zone mobilities in paper electrophoresis by EDWARD AND CRAWFORD[16].

In the following paragraphs, a new procedure for accomplishing the task is outlined. It is simple in concept, easily carried out from an experimental stand-point, theoretically sound and results in free solution values of migrants which agree very well with those determined directly.

EXPERIMENTAL AND THEORETICAL

Let us consider the case of a hypothetical migrant, M, bearing a negative charge, which has an experimentally determined ionographic mobility of 8.0 μ/sec, per unit potential gradient (V/cm), or 8.0 units, for short. Such a migrant might be an ampholyte, *e.g.*, protein, lipoprotein, amino acid, etc. present in a buffer solution whose pH is higher than that of the isoelectric point of the ampholyte. It is assumed that this is a meaningful mobility, in that the movement of the migrant has been shown to be linear with respect to time and potential[17]. The mobility as determined will be less than the migrant would be expected to have in the absence of a stabilizer. The difference can be attributed to (a) electro-osmosis, and (b) the barrier effect. The latter can be defined as the resultant vector of all physical and chemical forces, other than electro-osmosis, that hinder the free movement of a migrating moiety. Let us for the moment assume the the electro-osmotic effect is zero. Under these circumstances the mobility a migrant would have in the absence of a stabilizer can be determined with the aid of modified chromatographic experiments carried out under the same experimental conditions as those under which the ionographic measurements are to be made. In the experimental work reported here the following procedure was followed. The chromatographic migrant was applied to a wetted filter paper strip held in a horizontal position, in the ionographic apparatus[18]. The technique can be visualized more readily with the aid of Fig. 1. The filter paper was pre-marked at points A and B. The rack was positioned in the apparatus with only one end of the filter paper dipping in the buffer vessel. The buffer solution was allowed to flow up and across the paper until it reached point B. When the buffer had reached this point the migrant was applied as a thin streak across the paper strip at point A. The buffer solution was permitted to continue its flow until it had almost reached the other end of the paper strip. At this point the run was terminated and the buffer front, B^1, marked. The distance B to B^1 represents the movement of the solvent front while the distance A to A^1 represents the movement of the migrant. The distance A to A^1 divided by the distance B to B^1 will be referred to as the R_D or "relative distance" of the migrant. R_D, which will not exceed a value of 1, will be defined as the ratio of the distances of migrant movement to buffer movement for chromatographic experiments where the migrant is applied at a point which has already been wetted by the solvent. Since this technique represents a deviation from the usual chromatographic procedure where the migrant is applied to a dry paper surface, the term R_F has been avoided. In the case at hand, suppose the R_D of the migrant is 0.4.

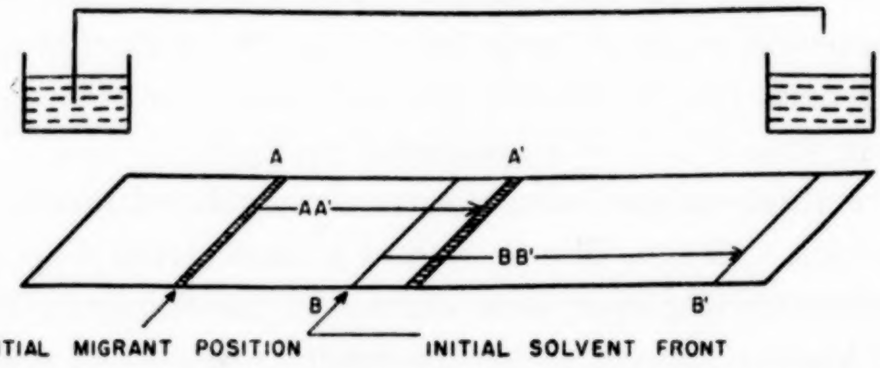

Fig. 1. Top: Arrangement of paper strip in ionographic apparatus for chromatography. Bottom: Diagramatic representation of chromatogram and derivation of R_D or relative distance.

$$\frac{\text{Migrant movement}}{\text{Solvent movement}} = \frac{AA'}{BB'} = R_D .$$

Since the ionographic mobility of the migrant was 8.0 units and the barrier effect, as measured by the value of $R_D = 0.40$, the mobility corrected for barrier effect but not for electro-osmosis, can be determined as follows.

40% of the mobility the migrant would have if there were no barrier effect corresponds to 8.0 units. Therefore, 100% of that same mobility should be given by: $100/40 \times 8.0$ or 20 units.

In the same fashion, the ionographic mobility of the electro-osmotic indicator, I, and its R_D value, may be determined. Let the mobility of the indicator be 3.2 units and the R_D be 0.8. The true electro-osmotic movement of I, corrected for the barrier effect, would then be 3.2/0.80 or 4.0 units. In the example above the electro-osmotic movement is toward the negative electrode while the movement of the migrant is toward the positive electrode. The resultant effect of electro-osmosis in this case is therefore to slow up the movement of the migrant, or, in other words, the true movement of the migrant corrected for electro-osmosis, would be 20 units *plus* 4 units or 24 units. This last value corresponds to the net movement of the migrant corrected both for barrier effect and for electro-osmosis, *i.e.*, it should correspond to the value of the migrant's mobility in the absence of a stabilizer, or to the so-called free solution value.

To be valid the method described here requires that the ionographic mobility of both the migrant and of the electro-osmotic indictor remain constant throughout the course of the experiment. For the results to be meaningful the movement of the migrant and of the electro-osmotic indicator must be shown to be linear with respect to time and to potential gradient, respectively. In the opinion of the authors, these criteria can, as a general rule, be fulfilled only with some form of ionographic apparatus in which the paper strip is held in a horizontal position.

To test the method outlined above, several crystallized proteins (obtained from Armour Laboratories, Chicago, Ill.), as well as human serum β-lipoproteins, isolated by differential ultracentrifugation, were chosen as the migrants. The β-lipoproteins consisted of the fraction having a density range, 1.019–1.063 g/ml. The materials whose free solution mobilities were determined were bovine plasma albumin, egg albumin, β-lactoglobulin, and human β-lipoproteins. The ionographic determinations were carried out under the same experimental conditions of buffer, pH, ionic strength and temperature as were the free solution experiments. Due to the relatively high ionic strength of the buffers, several modifications of the standard ionographic procedure described elsewhere[18], were made. Since the current drawn by the paper strips wetted with a buffer of ionic strength 0.1 or 0.2 would give rise to enough heat, and the concomitant evaporation of solvent, to prevent the attainment of a linear movement with time, these modifications were all aimed at reducing the development of heat in the strips to a minimum. The length of the paper strips was shortened to about 20 cm and four strips were mounted in the rack instead of the conventional seven strips. The potential gradient was reduced to about 1 V/cm.

TABLE I

Migrant	*Observed mobility* *cm^2/V-sec × 10^{-5}*	R_D	*Computed free solution mobility*	*Directly determined free solution mobility*
Glucose*	— 2.2	0.91	—	
Bovine plasma albumin*	4.2	0.96	6.8	6.86***
β-Lipoprotein*	0.54	0.45	3.6	3.5 (20)
Glucose**	— 2.5	0.87	—	
Bovine plasma albumin**	3.4	0.85	6.8	6.86***

Experimental conditions: Veronal buffer, pH 8.6, ionic strength 0.1, 2°, potential gradient 1 V/cm, time of run 16–20 h.

* Whatman No. 1 filter paper.

** Eaton-Dikeman No. 613 filter paper.

*** As determined by Armour Laboratories, Armour and Company, Chicago, Ill.

Glucose was used as the electro-osmotic indicator. Heating the strips in an oven at 100° for ½ h gave a yellow-brown zone for glucose. In later experiments with acetate buffers, the glucose was identified with 2,3,5-triphenyl-2H-tetrazolium chloride according to a method described by TREVELYAN and co-workers[19]. The data for bovine plasma albumin and human serum β-lipoproteins is given in Table I and the data for egg albumin and β-lactoglobulin is presented in Table II. From these data is can be seen that the free solution values obtained by the method outlined above are in close agreement with those determined directly.

TABLE II

Migrant	*Observed mobility* *cm^2/V-sec × 10^{-5}*	R_D	*Computed free solution mobility*	*Directly determined free solution mobility*
Glucose	— 0.80	0.76	—	
Egg albumin	4.5	0.92	5.9	5.69*
β-Lactoglobulin	4.4	0.96	5.6	5.62*

Experimental conditions: Phosphate buffer, pH 7.7, ionic strength 0.2, 2°, potential gradient 1 V/cm, time of run 16–20 h. Whatman No. 1 filter paper.

* As determined by Armour Laboratories, Armour and Company, Chicago, Ill.

To test the application of this method in the determination of isoelectric points and to explore the possibility that the barrier effect may be different for a given moiety on different sides of its isoelectric point, it was decided to determine the isoelectric points for human serum α- and β-lipoproteins. The α-lipoproteins were isolated by differential ultracentrifugation and represent the fraction having a density range, 1.063–1.210 g/ml.The isoelectric points were calculated from corrected mobilities obtained in acetate buffers at pH values of 4.0 and 5.9. By this method the isoelectric point of α-lipoprotein was found to be 5.3 and that of β-lipoprotein, 5.2. From the data in Table III, it can be seen that the barrier effect is quite different on different sides of the isoelectric points of α- and β-lipoproteins. Failure to take this fact into account would result in erroneous isoelectric points.

TABLE III

Migrant	*Observed mobility* *μ/sec/V/cm*	R_D	*Computed free solution mobility*
pH 4.0			
Glucose	— 0.13	0.92	—
β-Lipoprotein	— 0.056	0.03	— 1.76
α-Lipoprotein	— 0.091	0.05	— 1.66
pH 5.9			
Glucose	— 0.24	0.95	—
β-Lipoprotein	0.11	0.14	1.04
α-Lipoprotein	0.53	0.92	0.83

Experimental conditions: Acetate buffers, ionic strength 0.03, 25°, potential gradient 5 V/cm, atmosphere: water-saturated helium, Whatman No. 1 filter paper.

DISCUSSION

To simplify the application of the method outlined here for computing free solution electrophoretic mobilities, it would seem worthwhile to reiterate some of the principal ideas and to compress them into a form which can readily be used in practice.

Four measurements are required for each set of experimental conditions. These include the experimental determination of the ionographic mobility and R_D of the migrant and of the electro-osmotic indicator. For a *negatively* charged substance, *e.g.*, an ampholyte present in a buffer solution whose pH is greater than the isoelectric point of the migrant, the free solution mobility of the migrant, u_F, is given by:

$$u_F = \frac{\text{Mob. of migrant (experimental)}}{R_D \text{ of migrant}} + \frac{\text{Mob. of indicator}}{R_D \text{ of indicator}} \tag{a}$$

For a positively charged migrant, the situation can be stated as follows:

$$u_F = \frac{\text{Mob. of migrant (experimental)}}{R_D \text{ of migrant}} - \frac{\text{Mob. of indicator}}{R_D \text{ of indicator}} \tag{b}$$

The above relationships can be further simplified. Consider, for example, the case of a negatively charged migrant.

Let u_E = experimental mobility of migrant; u_I = experimental mobility of indicator.

Utilizing this notation, equation (a) above may be written:

$$u_F = \frac{u_E}{R_{DM}} + \frac{u_I}{R_{DI}} = \frac{R_{DI} \cdot u_E + R_{DM} \cdot u_I}{R_{DM} \cdot R_{DI}}$$

Finally, the following transformation may be made:

Let $m = R_D$ of migrant; $n = R_D$ of indicator; y = experimental mobility of migrant; z = experimental mobility of indicator.

Utilizing this notation, the following generalized expression may be written:

$$u_F = \frac{ny \pm mz}{mn} \tag{c}$$

If the migrant bears a negative charge, the "plus" sign is used; if a positive charge, the "negative" sign is used.

Applying equation (c) to the hypothetical migrant, bearing a negative charge, discussed earlier in this paper, the values to be inserted in the equation are:

$$\begin{aligned} n &= 0.8 \\ y &= 8.0 \\ m &= 0.4 \\ z &= 3.2 \end{aligned}$$

and the free solution mobility of the migrant, u_F ,is therefore given by:

$$u_F = \frac{0.8 \times 8.0 + 0.4 \times 3.2}{0.4 \times 0.8} = 24 \text{ mobility units}$$

SUMMARY

A practical experimental method for the conversion of ionographic mobilities of substances such as proteins and lipoproteins to free solution values has been presented. It is simple in concept, easily carried out from an experimental stand-point, theoretically sound and results in free solution values which agree very well with those determined directly. For the results to be valid, the ionographic mobility of both the migrant and the electro-osmotic indicator must remain constant throughout the course of the experiment. Four determinations are necessary for each set of experimental conditions. These include the experimental determination of the ionographic mobility and R_D, a modified R_F, of the migrant and of the electro-osmotic indicator. The measurements must be made under identical conditions of wetness and horizontality of the paper.

The method has been used to determine the free solution mobilities for bovine plasma albumin, human serum β-lipoproteins, egg albumin and β-lactoglobulin. The values obtained were in close agreement with those determined experimentally using classical free solution electrophoresis.

The isoelectric points of human serum α- and β-lipoproteins have also been determined and found to be in close agreement with previously reported values.

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FACTORS AFFECTING THE SEPARATION OF MILK PROTEINS AND PARTIALLY HYDROLYZED PROTEINS BY CONTINUOUS FLOW PAPER ELECTROPHORESIS

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The use of continuous paper electrophoresis in separating proteins has been described by GRASSMANN AND HANNIG[1], by DURRUM *et al.*[2,3], KARLER *et al.*[4], and by BRATTSTEN[5]. In these reports, instruments were described for continuous separation of proteins by paper electrophoresis. Several such instruments of this type are now commercially available. Most of the work on separations thus far reported has been on serum proteins and been concerned with the separation into fractions rather than in the production of pure fractions.

It has been reported by ZWEIG AND BLOCK[6] and MACRAE AND BAKER[7] that casein could be separated into its three components by zone paper electrophoresis. The latter authors determined the relative amounts of the three proteins by determining the amounts of bound dye or nitrogen analysis for each separate band.

The advantage of the continuous method over the zone method is that larger amounts can be separated and further tests of homogeneity can be made on the separated fractions.

BRATTSTEN[5] found that the resolving power of the cell he constructed was about one mobility unit. If this degree of separation of proteins is attainable, then it should be possible to separate α-, β-, and γ-casein by this method, which have mobilities of -6.8, -3.0, -2.0 units respectively at pH 8.4 and 0.1 ionic strength. The present paper describes the separation of these three caseins and the essential conditions of purifying proteins by this method.

MATERIALS AND METHODS

The continuous flow paper electrophoresis cell used was the Model CP manufactured by Beckman Instruments, Inc., Belmont, Calif.**.

Analysis of fractions from the curtain were made by measurements of biuret color, absorption at 278 mμ, or use of FOLIN-LOWRY reagent[8]. Biuret determinations on fractions were made using the reagent of BEISENHERZ[9]. 6 ml of biuret reagent was mixed with 1 ml of sample and after 30 min, the absorbance was read at 574 mμ against a reagent blank.

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* Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

** It is not implied the U.S.D.A. recommends the above company or its product to the exclusion of others in the same business.

Absorbance at 278 mμ was measured in Beckman Model DU spectrophotometer using 1 cm quartz cells. Since 0.025 ionic strength veronal has considerable absorbance near 278 mμ, a reagent blank was used.

Free electrophoresis was conducted in the 2 ml cells of the Spinco Model H Electrophoresis-Diffusion apparatus. The buffer used was pH 8.4, 0.1 ionic strength, 0.05 in veronal and 0.05 sodium chloride as previously used for the casein fractions in this laboratory[10].

β-Casein was prepared following the urea method of HIPP, GROVES AND McMEEKIN[10]. The trypsin hydrolysis was conducted as described by PETERSON, NAUMAN AND McMEEKIN[11].

Casein for the fractionation experiments was prepared by an acid precipitation at pH 4.6, followed by an extraction at pH 4.0 with acetic acid as described in a recent publication by McMEEKIN *et al.*[12]. By this method, a number of materials which amount to only 1 % of the total are removed.

Bovine plasma albumin, lot R370295A, was manufactured by Armour and Company.

EXPERIMENTAL

The Spinco Continuous Electrophoresis instrument is enclosed in a large plexiglass case to prevent rapid evaporation from the paper curtain. It was operated in a cold room at 5°, and chilled buffer from an 8 l vessel was circulated through a cooling plate ¼ inch behind the curtain before it was pumped into a reservoir at the top of the apparatus. An overflow diverted the largest part of this flow back into the reservoir. The height of the buffer in this reservoir governs the rate of feed by the upper siphon curtain which supplied buffer to the lower curtain where the electrophoretic separation is effected. The electrophoretic separation is made on a large sheet of heavy filter paper whose lower edge is divided into 32 drip points for the 32 collection tubes. At a point near the upper edge, the sample is applied, and the flow of buffer down the sheet carries the fraction into the collection tubes. The anode and cathode are two large packs of filter paper on the right and left edges of the lower sheet. In order to carry away the electrolysis products, two siphons carry buffer from the upper reservoir onto the top of these electrodes.The buffer flows down these electrodes and is recirculated.

As a preliminary to these experiments, a sheet of the filter paper supplied with the instrument was hung and wet with pH 8.6, 0.025 μ veronal buffer. At 1 inch intervals marked with pencil dots, a grid was constructed. After closing the face of the apparatus, equilibrium was established for 3 h, then with the buffer still flowing, a speck of solid dye was applied at each spot. At a downward flow rate of 8.0 ml/h (divided into 32 fractions), the side siphons were then adjusted for the most even collection in the sample tubes. The dye markers could not be used to follow buffer flow after voltage was applied to the sheet.

An estimate of spreading of a pure protein on the sheet was then obtained with a 2% bovine plasma albumin (BPA) solution in 0.025 μ pH 8.6 veronal buffer. With no voltage applied to the sheet, a thin trace of protein ran down from the point of application to the drip points and all the protein was contained in one of the 32 sample collection tubes. To make the trace visible for photography, the curtain was removed from the apparatus and quickly dried at 110° in a forced draft hot air oven. Then it was stained by the usual paper electrophoresis procedures using bromophenol blue[13].

A major variable is the rate of addition of the sample to be separated. The same degree of separation can be achieved by using repeated runs at a high sample addition rate, then taking a narrow fraction and rerunning the material. In this work we tried to determine the maximum separation obtainable by one passage down the curtain.

Bovine plasma albumin was used as the test substance to determine the effect of rate of feed on zone width. Using 0.01 μ pH 1.8 buffer, 6% acetic acid, 1% formic acid, a 2% solution of bovine plasma albumin, which had been previously dialyzed against the buffer, was put on the curtain. Fig. 1 shows the results. At a downward flow rate of 15.4 ml/h, 410 V at 30 mA produced a migration of about 18 cm (20 drip points). If the feed rate was 2 mg (0.1 ml) per h, the width of the trace expanded to 6 tubes; 6 mg (0.3 ml) per h widened the zone to 10 tubes. This shows clearly that for single pass separation, the feed rate must be kept low.

To find the relation between evaporation of buffer on the sheet and current flow, a low initial flow rate was used. With no voltage applied, 7.8 ml of buffer came down the curtain per hour. As the voltage and current were increased stepwise, the heat dissipated in the curtain finally rose to 65 W. At this point (84 mA and 780 V), the total flow had decreased to 6.4 ml/h, but the flow in the tubes was uneven, and the middle tube was dry.

Since the veronal in the buffer is non-volatile, 25 ml portions of the buffer and the samples were evaporated at 110°. The surprising results are shown in Table I.

TABLE I

Current through curtain mA	*Buffer flow ml/h*	*Av. conc. of buffer μ*
14	8.9	0.025
56	7.8	0.052
84	6.4	0.095

Evidently the wicks on the side of the curtain are acting as a source of buffer to replace the water loss from the heated curtain. Approximately four times as much buffer volume is entering the sheet as is dripping from the lower points. The use of this much current will greatly distort the separations since the lateral flow of buffer into the sheet will compress the pattern and oppose the migration due to the mobility of the components.

The choice of working conditions may then be summarized. For the best single passage separation, flow of buffer down the curtain is set at the lowest value which does not distort the pattern of separation. Voltage (and wattage) are kept low, below 20 watts. Feed rate is held at about 2–4 mg of protein per hour.

The separation of a 50–50 mixture of α-lactalbumin and β-lactoglobulin on the acidic or basic side of the isoelectric point is a good test of the resolving power of the

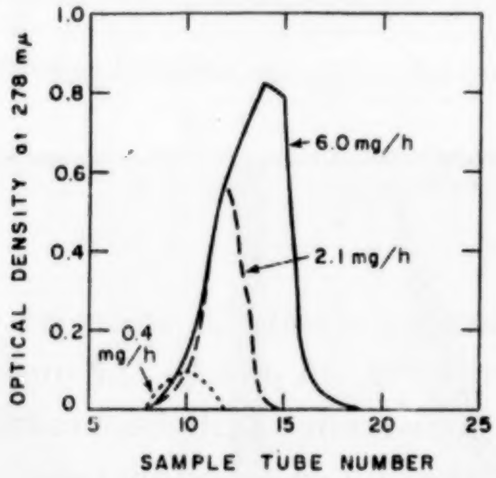

Fig. 1. Effect of sample feed rate on spread of bovine plasma albumin in collection tubes.

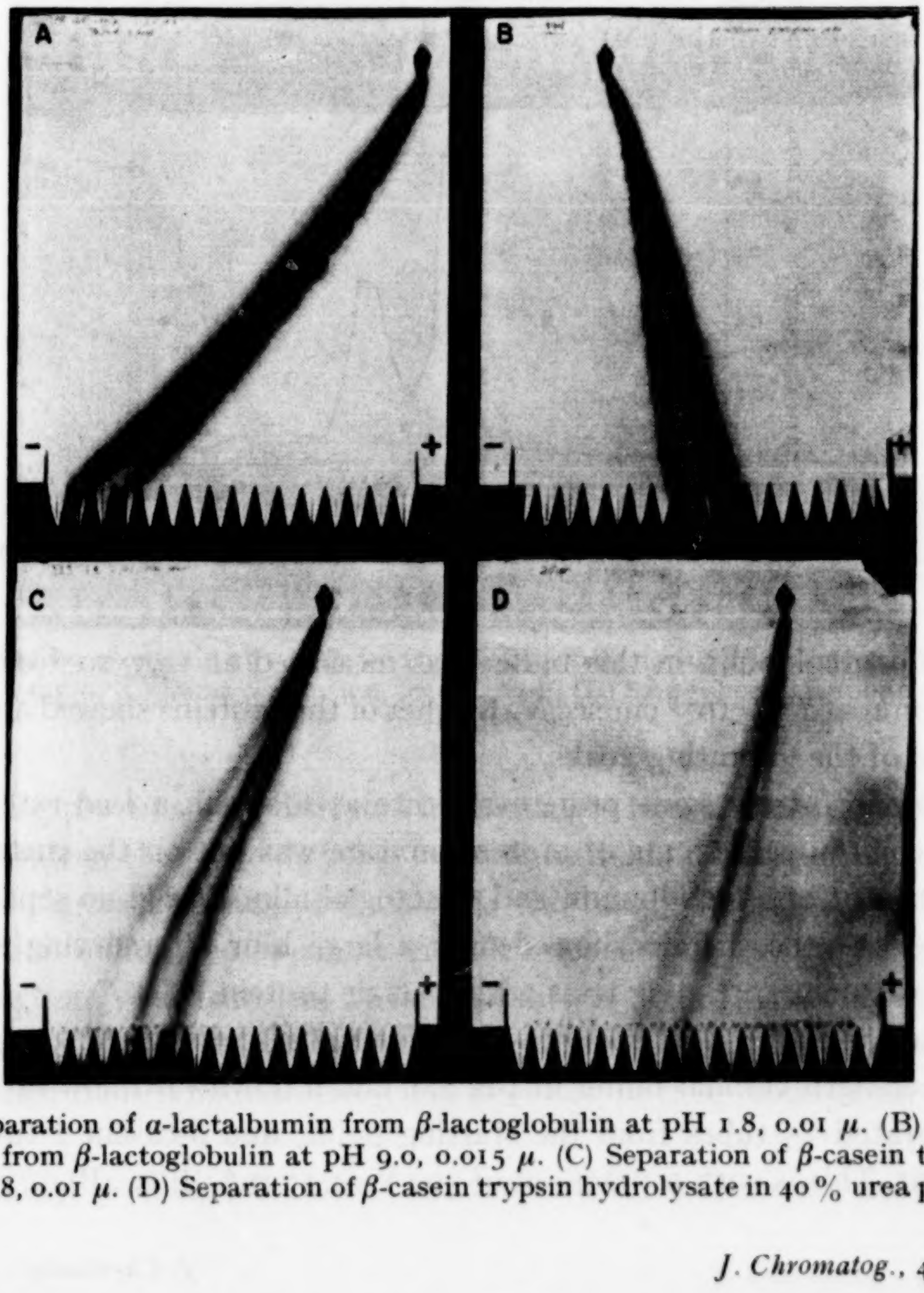

Fig. 2. (A) Separation of α-lactalbumin from β-lactoglobulin at pH 1.8, 0.01 μ. (B) Separation of α-lactalbumin from β-lactoglobulin at pH 9.0, 0.015 μ. (C) Separation of β-casein trypsin hydrolysate at pH 1.8, 0.01 μ. (D) Separation of β-casein trypsin hydrolysate in 40% urea pH 2.0, 0.01 μ.

apparatus. At pH 1.8, in 0.01 μ acetic acid–formic acid buffer, the two proteins have to flow nearly across the sheet before any separation is visible. On the alkaline side, a lesser flow is sufficient. The tabulated mobilities for free electrophoresis are as follows:

TABLE II

MOBILITIES OF β-LACTOGLOBULIN AND α-LACTALBUMIN IN ACID AND BASIC BUFFERS AT 0.1 μ

	pH 2.0	*pH 8.5*
β-Lactoglobulin	+ 10	— 5.0
α-Lactalbumin	+ 7.8	— 4.2

These indicate that the separation should be easier at pH 2.0 than 8.5. However, the reverse is true, as shown in Fig. 2A and B. The quality of the separation at pH 9.0 in 0.015 ionic strength triethylamine carbonate buffer is shown in Fig. 3 where the ultraviolet absorption of the sample collection tubes is plotted. Of 100 mg of the mixture, 36 and 35 mg were collected in tubes 15–18 and 20–24. Paper strip electrophoresis of the lyophilized products showed that the separation was clean.

Although the values were obtained for 0.1 ionic strength solutions, the same ratio of mobilities exist in the 0.01 ionic strength pH 2 buffer used on the curtain. The mobility of β-lactoglobulin in this buffer was measured at $14.7 \cdot 10^{-5}$ cm/sec/V, that of α-lactalbumin at $11.5 \cdot 10^{-5}$ cm/sec/V. Neither of the proteins showed any abnormalities in shape of the migrating peaks.

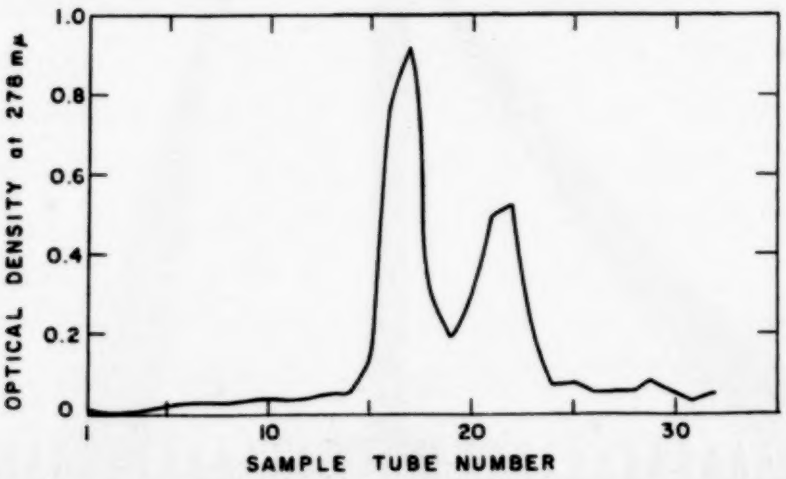

Fig. 3. Separation of α-lactalbumin and β-lactoglobulin in pH 9.0, 0.015 μ triethylamine carbonate buffer.

The behavior of the same protein mixture at too high a feed rate is shown in Fig. 4A. At 1 ml/h feed, 20 mg of protein mixture was put on the curtain per hour. This large amount of α-lactalbumin and β-lactoglobulin showed no separation in the sample tubes and the curtain showed only a large blur after drying and staining. This agreed with our spreading tests with a single protein.

The separation of α-, β-, and γ-casein was next attempted. At 3 mg/h feed rate, 0.025 ionic strength veronal buffer at pH 8.6, and a downward flow rate of 13 ml/h, α-casein migrated 15 tubes from the starting point, and β-casein 7 tubes from the starting point. This is with a potential of 300 V at 17 mA (Fig. 4B).

This could be improved further, as shown in Fig. 4C. Here the counterosmotic flow was shown by adding 1 % lactose to the 2 % casein feed solution. On drying, the lactose reacted with the buffer to produce a tan color which was outlined in pencil to show in the photographs. Here the feed rate was reduced to 2 mg/h and the cleanness of the separation was improved.

Finally, as shown in Fig. 4D, the downward flow of buffer was decreased to 10 ml/h with other conditions the same. Here owing to the counterosmotic flow, the paths of the migrating proteins were distorted from straight lines. Further decrease in buffer flow would result in the proteins being swept into the lefthand side-electrode.

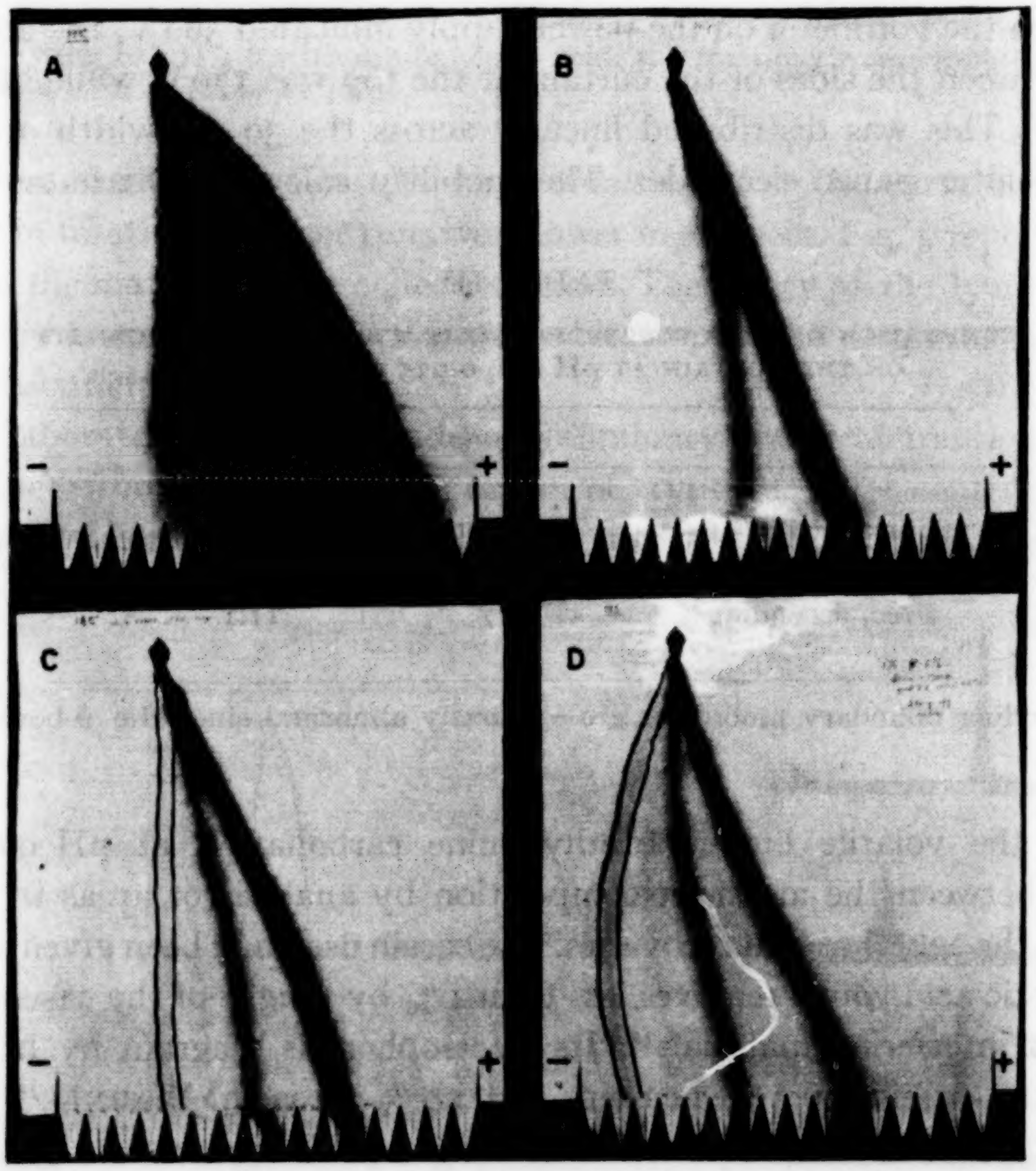

Fig. 4. (A) Flooding of curtain at 1 ml/h feed rate. (B) Separation of whole casein into α-, β-, and γ-fractions, feed 3 mg/h. (C) Same separation, feed 2 mg/h. (D) Same separation, buffer flow lowered to 10 ml/h.

When whole casein is analyzed by zone electrophoresis at pH 8.6 in 0.05 ionic strength veronal buffer, the apparent mobility depends on the paper wetness. In a thin paper such as Whatman No. 1, the mobility is about 54 % and in the thicker Whatman No. 3 paper, the mobility is about 71 % of the value in free solution. The curtain is thicker than Whatman No. 3 and is wetter, so the mobility may be expected to be higher.

The mobility on the curtain can be calculated if we know the field strength in volts/cm and the length of time the protein is in the electrical field on the curtain[14]. From the rate of buffer flow into the sample tubes and the average buffer content of the curtain (determined by weighing), it appears that the protein is in the field for 6 h. This agrees with the time for the first appearance of protein in the sample tubes after the protein is started on the curtain. This 6 h holds, of course, only at the buffer flow rate of 10.6 ml (in 32 tubes) and for the applied voltage of 300 V at 17 mA. With the front of the cabinet removed after the curtain had been running for some time, a vacuum tube voltmeter was used to measure the actual voltage distribution on the curtain. When the voltmeter on the power supply indicated 300 V, the actual voltage difference between the sides of the curtain at the top was 170 V, while at the bottom it was 233 V. This was distributed linearly across the 30 cm width of the curtain between the filter paper electrodes. The mobility calculations are summarized in Table III.

TABLE III

COMPARISON OF FREE ELECTROPHORESIS MOBILITIES AND MOBILITY ON THE CURTAIN AT pH 8.6, 0.025 IONIC STRENGTH

	α-Casein	*β-Casein*
Curtain	$9.0 \cdot 10^{-5}$	$4.8 \cdot 10^{-5}$
Free, descending	10.05	5.01
Free, ascending*	16.7	11.1

* The ascending boundary mobilities are obviously abnormal since the δ-boundary is much larger.

The use of the volatile buffer, triethylamine carbonate[15], at pH 9.0 enabled a comparison between the apparent composition by analysis of areas in free electrophoresis and the actual weight recoveries. The casein used had been given an extraction with 1 % acetic acid which removes less than 1 % by weight of the casein, containing a number of interfering materials[12]. Its electrophoresis diagram by fringe analysis showed 72 % α-casein, 26.3 % β-casein, and 1.7 % γ-casein. WARNER[16] first demonstrated the complexing of β-casein in the α-casein peak area.

When fed onto a curtain at a high feed rate, 4 mg/h and a downward buffer flow of 15 ml/h, 360 V sufficed to spread the protein out nearly across the sheet. The buffer was 0.015 ionic strength and pH 9.0 triethylamine carbonate. The current was only 18 mA. As the buffer did not absorb UV light at 278 mμ, a comparison could be made of weight recovery and UV absorption using the extinction coefficients supplied by HIPP[17] of 1.024, 0.497, and 0.500 for α-, β- and γ-casein respectively.

The weight recoveries after the flow paths had been established for three days period are given in Table IV.

MACRAE AND BAKER[7] have shown that whole casein, analyzed by paper electrophoresis after correcting for the different dye binding capacities, consisted of 60 % α-casein, 32 % β-casein and 9 % γ-casein. MCMEEKIN, HIPP AND GROVES[12] have shown similar weight recovery of α-casein in a quantitative separation.

TABLE IV

CURTAIN SEPARATIONS COMPARED WITH ELECTROPHORESIS AREA OF STARTING MATERIALS

	% of total		
	α-casein	*β-casein*	*γ-casein*
Weight recovery*	58.6	35.4	6.0
UV absorption	57.8	37.5	5.8
Area analysis of starting material	72.0	26.3	1.7

* After adjusting to pH 4.6 with dilute acetic acid, the fractions were lyophilized in weighed flasks, then dried 16 h *in vacuo* at 40°.

It must be noted that these results were obtained after the curtain was saturated with the casein fractions. Earlier runs were lower in α-casein. Fig. 5 represents the UV absorption of the material in the collection tubes. The purity of the lyophilized α- and β-casein fractions are shown in Fig. 6 as the electrophoresis diagrams in pH 8.6, 0.1 ionic strength buffer.

In this laboratory we have made a preliminary chemical fractionation of the peptides formed from β-casein by the action of trypsin[11]. After centrifuging off the insoluble material formed at pH 7.0 after 20 min of trypsin action, the solution is acidified to pH 4.7. The precipitate at this point amounts to about 20 % of the starting material and contains 5 major component peptides with mobilities — 1.8, — 2.4, — 3.0, — 5.1, and — 9.4 at pH 9.0, 0.1 ionic strength in the Tiselius apparatus.

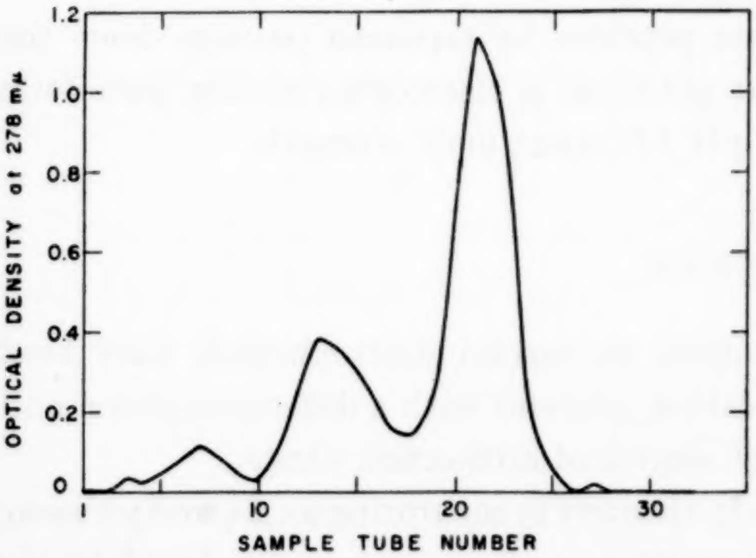

Fig. 5. Separation of α-, β-, and γ-casein using pH 9.0, 0.015 μ triethylamine carbonate buffer.

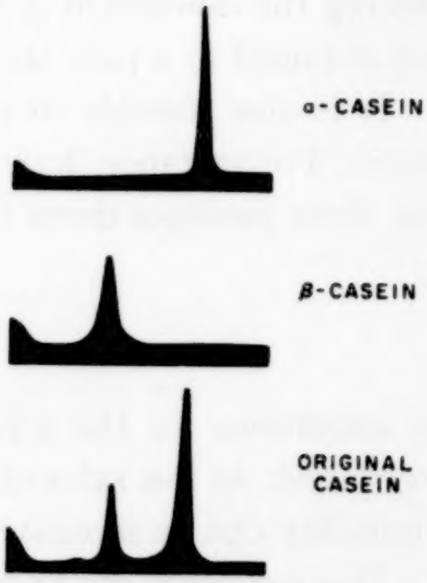

Fig. 6. Free electrophoresis of α- and β-casein fractions separated by curtain electrophoresis.

If this material is separated on the curtain using a 1 % formic acid–6 % acetic acid buffer, at a downward flow of 7 ml/h (total in 32 tubes) 240 V at 17.5 mA gives a visible separation of four components on the dyed sheet (Fig. 2C). The phosphopeptone, — 9.4 mobility, dissolves off the sheet in the bromophenol blue dyebath. However, on subjecting the lyophilized fractions to analytical paper electrophoresis, it is seen that only two components, mobility — 1.8 and — 9.4, have been obtained pure. This is despite the apparent cleanness of the separation as shown by analysis of the protein content of the tubes with biuret reagent (Fig. 7). GRANZER[18] has shown that the products of chymotryptic digestion of insulin also are complex although they migrate as single lines. By using 40% urea or 4% phenol in the acid buffer, he was able to separate the mixture of peptides with one operation. Use of the urea as a solvent in the curtain apparatus reduces the number of tubes over which the fractions were spread, so the first fractionations were not successful. However, by still further reducing the downward flow of buffer, peptides with mobilities of — 2.4 and — 3.0 were obtained in a pure state.

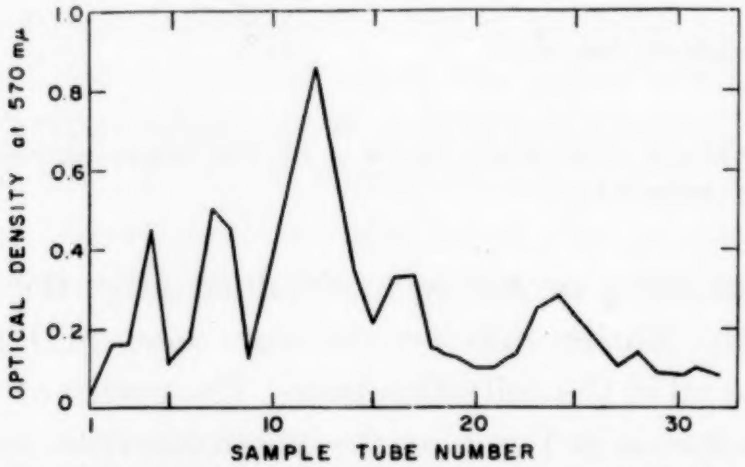

Fig. 7. Separation of peptides produced from β-casein by trypsin action, pH 1.8, 0.01 μ.

It is also possible to purify the other peptides by repeated passage down the curtain. For instance, fraction — 2.4 was obtained in electrophoretically pure form after three passages down the curtain at pH 8.6, 0.025 ionic strength.

SUMMARY

The conditions for the separation of proteins by curtain electrophoresis have been determined. At low rates of sample application, proteins with a difference of one unit of mobility can be separated unless a high degree of interaction exists.

This method is shown to be particularly effective in separating α-, β-, and γ-casein, a mixture of α-lactalbumin and β-lactoglobulin, as well as peptides produced by the action of trypsin on β-casein.

The amounts of material which can be purified by curtain electrophoresis are small, however, they are adequate to produce enough material for characterization of proteins and peptides by modern analytical methods.

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THE SEPARATION OF THE LONG CHAIN FATTY ALDEHYDES BY GAS–LIQUID CHROMATOGRAPHY

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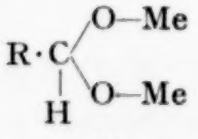
Investigations of the fatty acid and fatty aldehyde composition of the plasmalogens, a class of phospholipids present in a variety of animal tissues demand a rapid method for the qualitative and quantitative analysis of mixtures of long chain fatty aldehydes.

The separation and identification of fatty acid methyl esters by gas–liquid chromatography is now well established and reliable retention data[1–3] have been published for a large number of fatty acids of known structure. This paper describes the separation of the long chain fatty aldehydes using the same conditions as those required for separation of the acid methyl esters.

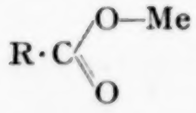
The long chain fatty aldehydes are rather unstable compounds which tend to polymerize over a period of time to give mixtures of monomer, dimer and trimer forms. This instability makes the aldehyde itself unsuitable for gas–liquid chromatography. Fortunately the aldehyde can easily be converted to a derivative, the aldehyde dimethyl acetal, which is quite stable and has the added advantage of a much lower boiling point.

An aldehyde dimethyl acetal (I) and its corresponding acid methyl ester (II)



$R\cdot CH(O\text{–}Me)_2$

(I)



$R\cdot C(=O)\text{–}O\text{–}Me$

(II)

R = long aliphatic chain

differ only in the degree of polarity exhibited by the end groups, the polar effect of the methyl ester being the stronger. Therefore the non-polar and polar stationary phases, which give excellent separations of the fatty acid methyl esters, should also be suitable for separating mixtures of aldehyde dimethyl acetals; and in fact mixtures of esters and mixtures of acetals can be separated with comparable efficiency under identical conditions.

EXPERIMENTAL

Materials

Several standard aldehydes were synthesised by the method of WEGAND *et al.*[4] from the corresponding acid chloride. The yields of palmitaldehyde (80 %), stearaldehyde

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J. Chromatog., 4 (1960) 52–59

(80 %) and oleic aldehyde (70 %) were very satisfactory. The yield of linoleic aldehyde was only 50 % whereas that of linolenic aldehyde was only 10 %. The low yield of linolenic aldehyde was probably due to the fact that since only a small amount of starting material (0.5 g linolenic acid) was available it was not possible to purify the products of one stage of the reaction before proceeding with the next. Myristaldehyde was a gift from Dr. G. SLOANE-STANLEY.

All the aldehydes were converted to their dimethyl acetal derivatives by refluxing for 2 hours with 2 % anhydrous methanolic HCl (solvent: solute, 20:1 v/v). The conversion was quantitative (> 95 %). The methanolic solution was allowed to cool and the HCl neutralized by the addition of a slight excess of anhydrous sodium carbonate. The acetals were extracted from the methanol with petroleum ether (b.p. 40–60°). The petroleum ether solution was shaken with saturated sodium metabisulphite solution to remove unchanged aldehyde and then with water until neutral. The solution was dried over anhydrous sodium sulphate and the aldehyde dimethyl acetal recovered by removal of the solvent under reduced pressure.

A possible side product of the aldehyde synthesis was the corresponding acid, which would now be present as methyl ester mixed with the aldehyde dimethyl acetal. The acetal was therefore refluxed for 2 h with 0.5 *N* methanolic sodium hydroxide to convert the acid to its sodium salt. The solution was cooled, diluted with water, and the acetal was extracted with petroleum ether (b.p. 40–60°). The petroleum ether was washed with water and dried over anhydrous sodium sulphate. The acetal was recovered by removing the solvent under reduced pressure and finally purified by redistillation under vacuum.

The dimethyl acetals of mixtures of a wide range of aldehydes isolated from natural sources[5] (*e.g.* ox heart, pig heart, ox spleen and ram semen) were prepared in the same way. The aldehydes from natural sources were always contaminated with small amounts of fatty acid and needed purifying by refluxing with methanolic sodium hydroxide.

Hydrogenation of aldehyde dimethyl acetals was carried out in ethyl alcohol (solvent: solute, 100:1 v/v) using a platinum oxide catalyst. It was sometimes necessary to add up to 20 % of chloroform to keep the acetals dissolved throughout the reaction period. Bromination of aldehyde dimethyl acetals was carried out by dissolving them in dry ether (ether: acetals, 10:1 v/v) at 0° and adding bromine until a permanant coloration was produced. The solution was allowed to stand for 15 minutes and then the solvent and excess bromine was removed on a rotary evaporator.

Columns

The gas–liquid chromatographic apparatus was a laboratory-constructed model using the argon β-ray ionization detector designed by LOVELOCK, JAMES AND PIPER[6]. All columns were of glass 4 ft. long and 4 mm internal diameter. The stationary phases used were Apiezon L grease[7] (1.8 g to 7 g celite), Reoplex 400[8] (2.1 g to 7 g celite) and polyethylene glycol adipate[3] (2.1 g to 7 g celite). The celite (100–120 mesh) was prepared by the method of JAMES AND MARTIN[9] and for use with Apiezon L grease was pretreated with alcoholic alkali[10]. The celite used with both polyesters was not pretreated with alkali.

RESULTS

(*a*) *Relative retention volumes of the aldehyde dimethyl acetals*

The carbon numbers of each homologous series were plotted against the logarithms of the retention volumes[9] relative to stearaldehyde dimethyl acetal on both Apiezon L and Reoplex 400 (Fig. 1). The closed symbols represent the plots of pure synthetic aldehyde standards of known structure. A number of straight chain saturated and mono-unsaturated aldehydes found in mixtures isolated from natural sources were identified beyond reasonable doubt by the retention volumes of their dimethyl acetals (represented by the open symbols in Fig. 1) relative to the standard compounds both on the non-polar Apiezon L and the polar Reoplex 400. To confirm the peak positions of the unsaturated aldehydes the mixtures of dimethyl acetals were chromatographed both as isolated and after hydrogenation.

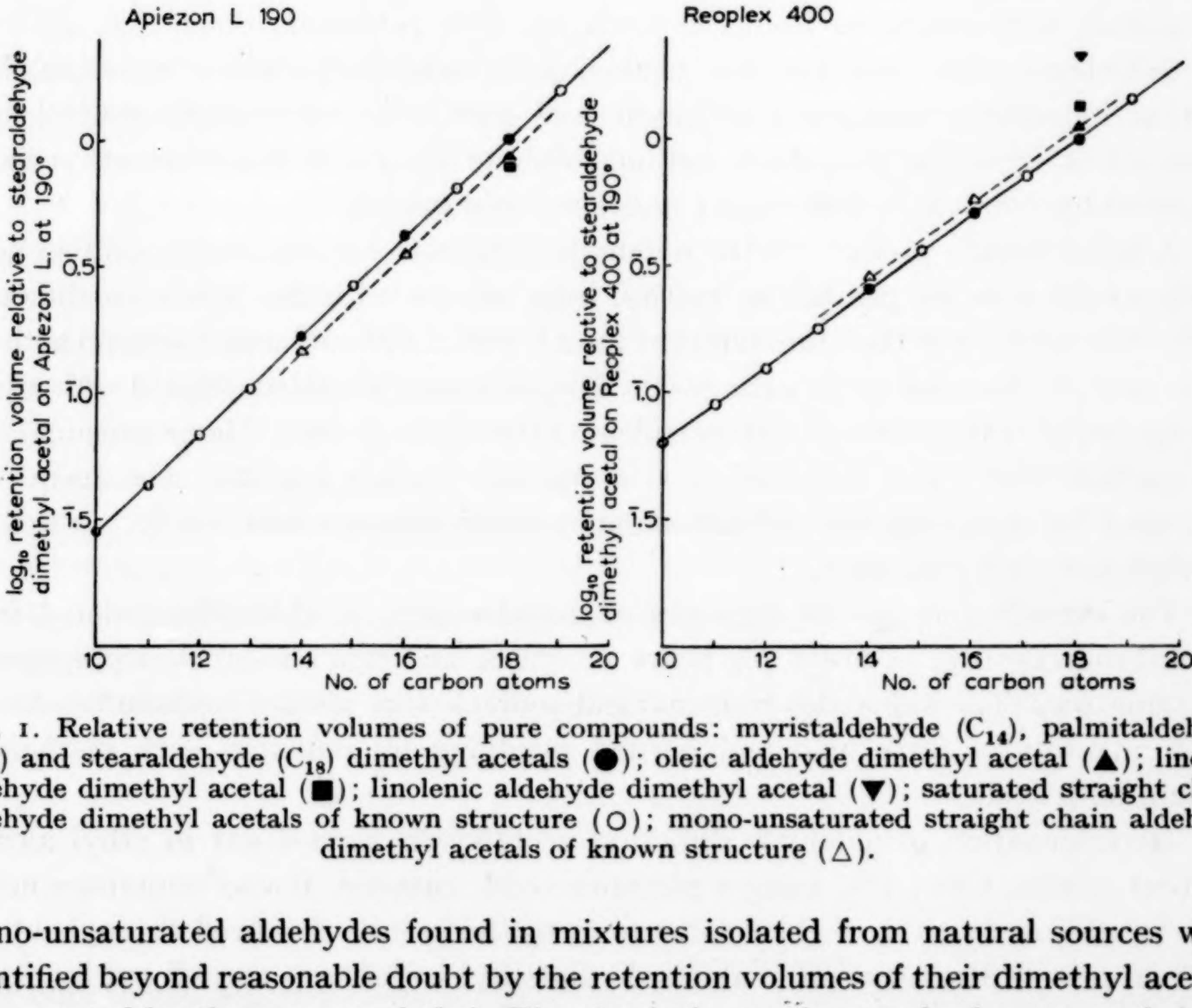

Fig. 1. Relative retention volumes of pure compounds: myristaldehyde (C_{14}), palmitaldehyde (C_{16}) and stearaldehyde (C_{18}) dimethyl acetals (●); oleic aldehyde dimethyl acetal (▲); linoleic aldehyde dimethyl acetal (■); linolenic aldehyde dimethyl acetal (▼); saturated straight chain aldehyde dimethyl acetals of known structure (○); mono-unsaturated straight chain aldehyde dimethyl acetals of known structure (△).

Bromination of the mixtures of dimethyl acetals as a means of identifying unsaturated aldehydes was carried out as an alternative to hydrogenation. It was useless for this purpose because when a brominated sample of mixed saturated and unsaturated aldehyde dimethyl acetals was chromatographed the peaks which represented saturated compounds had disappeared as well as those of the unsaturated compounds. This indicated that the bromine had reacted with the saturated dimethyl acetals to produce non-volatile derivatives and the results with brominated pure palmitaldehyde dimethyl acetal provided the necessary confirmation. Bromination is therefore not suitable as a means of identifying unsaturated components but it provides a useful check on the purity of the dimethyl acetals, for no peaks will occur on chromatographing a pure sample of acetals after successive hydrogenation and bromination, but any contaminating acid methyl esters in a sample so treated will appear as peaks.

(*b*) *A comparison of the relative retention volumes of aldehyde dimethyl acetals with acid methyl esters*

The retention volumes of a series of aldehyde dimethyl acetals relative to stearaldehyde dimethyl acetal were compared with the retention volumes of corresponding acid methyl esters relative to methyl stearate by chromatographing both types of compound under identical conditions (Fig. 2 and Table I). The dimethyl acetals have a

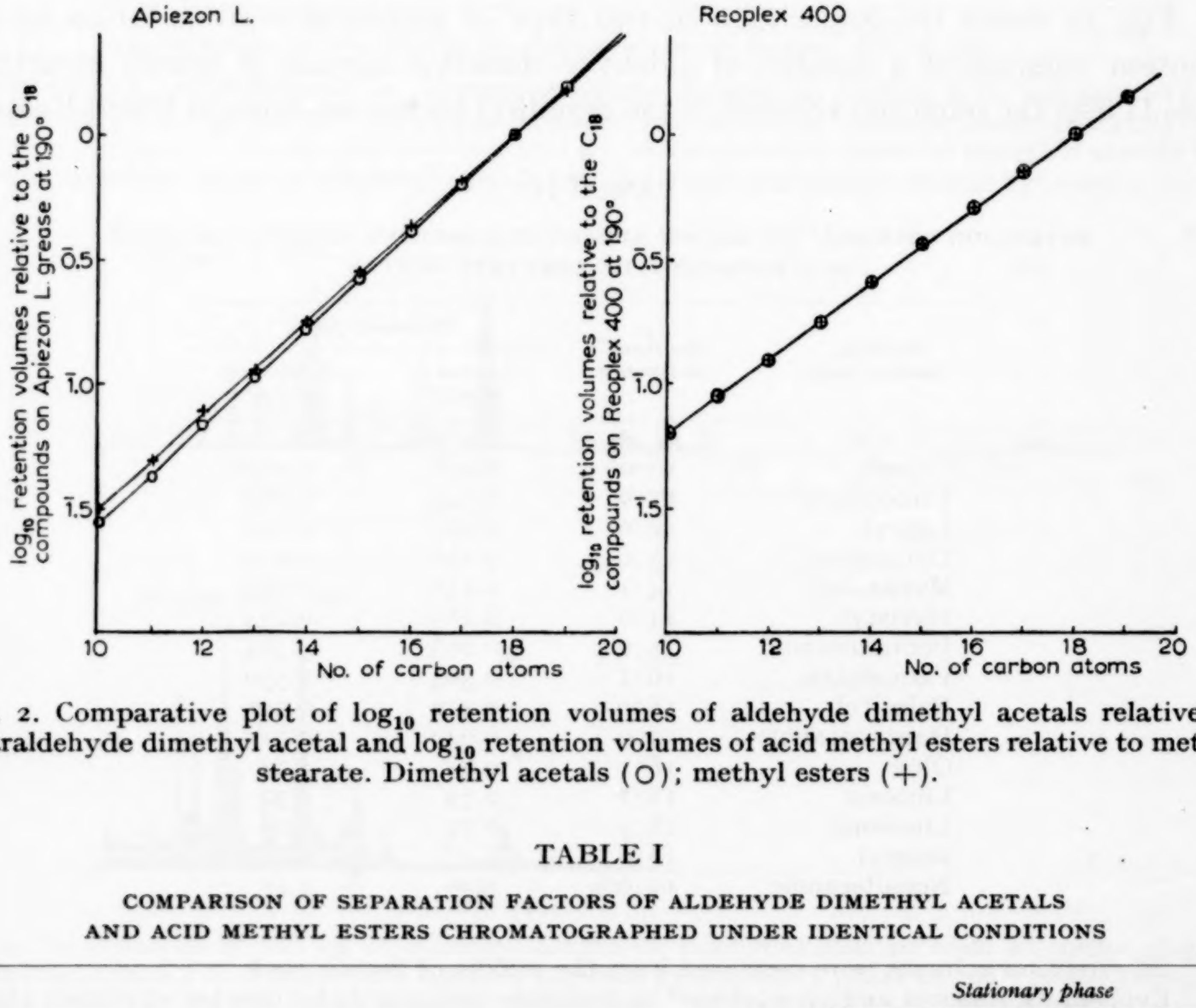

Fig. 2. Comparative plot of $\log_{10}$ retention volumes of aldehyde dimethyl acetals relative to stearaldehyde dimethyl acetal and $\log_{10}$ retention volumes of acid methyl esters relative to methyl stearate. Dimethyl acetals (○); methyl esters (+).

TABLE I

COMPARISON OF SEPARATION FACTORS OF ALDEHYDE DIMETHYL ACETALS AND ACID METHYL ESTERS CHROMATOGRAPHED UNDER IDENTICAL CONDITIONS

		Stationary phase	
		Apiezon L at 190°	*Reoplex 400 at 190°*
Retention volume of methyl stearate relative to stearaldehyde dimethyl acetal		0.77	1.19
—CH_2-separation factor[9]	aldehyde dimethyl acetals	1.57	1.40
	acid methyl esters	1.55	1.40

slightly better separation factor on Apiezon L grease than the methyl esters but on Reoplex 400 the factor is the same. The retention volume of an aldehyde dimethyl acetal compared with that of the corresponding acid methyl ester is higher on the non-polar Apiezon L stationary phase and lower on the polar Reoplex 400 phase showing quite clearly the effect of a less polar end group (*i.e.* the dimethyl acetal) on the retention volume of a straight long chain carbon compound on non-polar and polar stationary phases.

(*c*) *Identification of unknown compounds*

JAMES[3] has shown that by plotting $\log_{10}$ relative retention volumes for a variety of fatty acids on Apiezon L against the values obtained with a polyester stationary phase points are obtained which lie on a series of straight parallel lines each line corresponding to a given type of acid. For example all the straight chain fatty acids fall along one line and the mono-unsaturated acids fall on another parallel line.

Fig. 3a shows the application of this type of graphical representation to the retention volumes of a number of aldehyde dimethyl acetals of known structure. Table II lists the retention volumes of the dimethyl acetals on Apiezon L and Reoplex 400. Some of the mixtures of aldehydes isolated from natural sources are very complex (Fig. 4) and it is not possible to identify all the components with certainty. The straight chain saturated and unsaturated aldehydes can be identified with certainty from their retention volumes on the two stationary phases relative to known com

TABLE II

RETENTION VOLUMES* OF KNOWN ALDEHYDE DIMETHYL ACETALS RELATIVE TO STEARALDEHYDE DIMETHYL ACETAL

Aldehyde, familiar name	*Shorthand** designation*	*Stationary phase*	
		Apiezon L at 190°	*Reoplex 400 at 190°*
Capryl	10:0	0.028	0.064
Undecanoic	11:0	0.043	0.089
Lauryl	12:0	0.067	0.124
Tridecanoic	13:0	0.106	0.177
Myristoleic	14:1	0.150	0.280
Myristyl	14:0	0.167	0.254
Pentadecanoic	15:0	0.263	0.364
Palmetoleic	16:1	0.364	0.570
Palmityl	16:0	0.412	0.506
Heptadecanoic	17:0	0.640	0.715
Oleic	18:1	0.84	1.12
Linoleic	18:2	0.79	1.34
Linolenic	18:3	0.79	1.68
Stearyl	18:0	1.0	1.0
Nonadecanoic	19:0	1.56	1.41

* All retention volumes were measured from the middle of the air peak.

** Evolved by AHRENS and co-workers[11] to designate compounds by number of carbon atoms, number of double bonds and presence of chain branching, *e.g.* br. 16:1 represents a branched chain mono-unsaturated C_{16} compound.

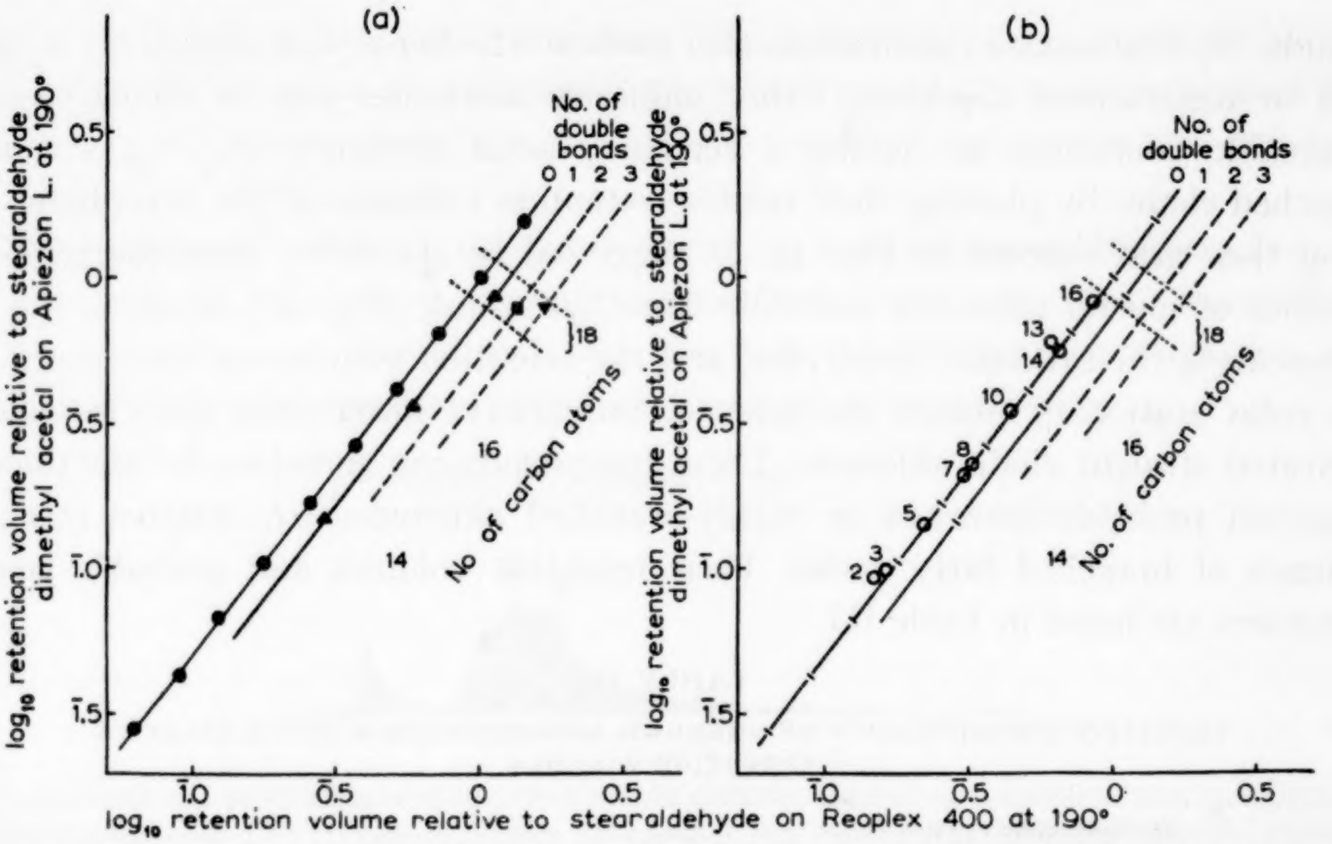

Fig. 3. (a) $\log_{10}$ relative retention volumes of dimethyl acetals on Apiezon L plotted against $\log_{10}$ relative retention volumes on Reoplex 400. Straight chain saturated aldehyde dimethyl acetals (●); mono-unsaturated aldehyde dimethyl acetals (▲); di-unsaturated aldehyde dimethyl acetals(■); tri-unsaturated aldehyde dimethyl acetals (▼). (b) Unknown aldehyde dimethyl acetals (○).

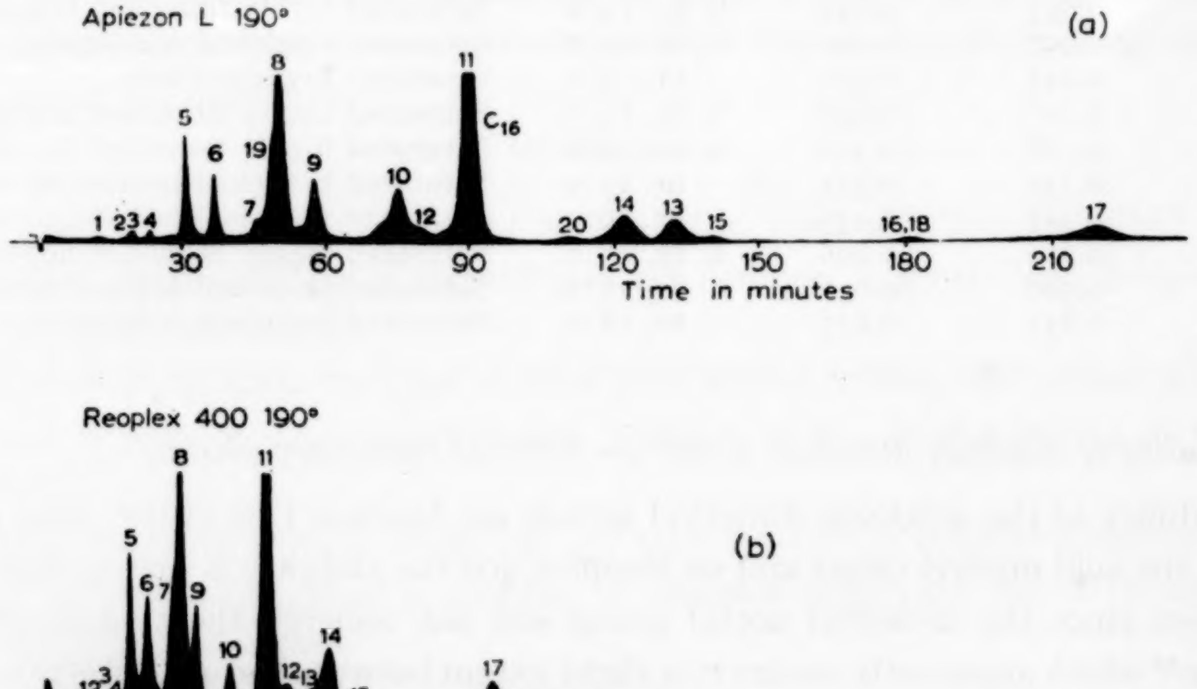

Fig. 4. (a) Analysis of 100 μg of dimethyl acetals of aldehydes isolated from ox-spleen choline plasmalogen on a 4 ft. column with Apiezon L grease as stationary phase at 190°. Argon pressure, column inlet, 103 cm Hg, outlet pressure, atmospheric. (b) Analysis of 100 μg of dimethyl acetals of aldehydes isolated as above on a 4 ft. column with Reoplex 400 as a stationary phase at 190°. Argon pressure, column inlet 72.5 cm Hg, outlet pressure, atmospheric.

Peaks: (1) *n*-dodecanoic; (2) probably highly-branched tridecanoic; (3) probably branched tridecanoic; (4) *n*-tridecanoic; (5) probably branched tetradecanoic; (6) *n*-tetradecanoic; (7) probably highly-branched pentadecanoic; (8) probably branched pentadecanoic; (9) *n*-pentadecanoic; (10) probably branched hexadecanoic; (11) *n*-hexadecanoic; (12) palmitoleic; (13) probably highly-branched heptadecanoic; (14) probably branched heptadecanoic; (15) *n*-heptadecanoic; (16) probably branched octadecanoic; (17) *n*-octadecanoic; (18) oleic; (19) probably highly-branched pentadecanoic; (20) unknown.

pounds. Hydrogenation experiments also confirm whether a peak represents a saturated or unsaturated aldehyde. Other unknown aldehydes can be identified with reasonable confidence as having a certain general structure (*e.g.* C_{16} saturated branched chain) by plotting their relative retention volumes on the two phases and using the "grid" shown in Fig. 3a, as suggested by JAMES[3]. Thus the retention volumes of several unknown aldehyde dimethyl acetals (Fig. 3b) lie along the line representing the saturated aldehydes, and the retention volumes on both polar and non-polar stationary phases are near to, but always lower than a corresponding saturated straight chain aldehyde. These compounds can therefore be identified as saturated probably-branched or highly-branched aldehydes (*cf.* relative retention volumes of branched fatty acids). Their retention volumes and probable general structures are listed in Table III.

TABLE III

TENTATIVE IDENTIFICATION OF UNKNOWN ALDEHYDES FROM THEIR RELATIVE RETENTION VOLUMES

Peak No. (Fig. 4)	*Retention volumes relative to stearaldehyde dimethyl acetal*		*Shorthand designation*	*Aldehyde*
	Stationary phase			
	Apiezon L at 190°	*Reoplex 400 at 190°*		
2	0.095	0.146	h. br. 13:0	Saturated highly branched tridecanoic
3	0.098	0.160	br. 13:0	Saturated branched tridecanoic
5	0.141	0.217	br. 14:0	Saturated branched tetradecanoic
7	0.210	0.310	h. br. 15:0	Saturated highly branched pentadecanoic
19	0.218	0.310	h. br. 15:0	Saturated highly branched pentadecanoic
8	0.230	0.325	br. 15:0	Saturated branched pentadecanoic
10	0.344	0.435	br. 16:0	Saturated branched hexadecanoic
13	0.610	0.606	h. br. 17:0	Saturated highly branched heptadecanoic
14	0.560	0.647	br. 17:0	Saturated branched heptadecanoic
16	0.835	0.855	br. 18:0	Saturated branched octadecanoic

(*d*) *Stability of aldehyde dimethyl acetals on different stationary phases*

The stability of the aldehyde dimethyl acetals on Apiezon L is of the same order as that of the acid methyl esters and on Reoplex 400 the stability is higher than that of the esters since the dimethyl acetal group will not undergo the transesterification reaction[12] which apparently occurs to a slight extent between the methyl esters and the ester groups of the stationary phase. Accordingly reliable quantitative data can be obtained by measurement of peak areas obtained from chromatographing mixtures on either phase.

Experiments were carried out using a third stationary phase polyethylene glycol adipate prepared as described by JAMES[3]. This stationary phase, unlike Reoplex 400 does not bleed from the column at high temperatures and so more efficient columns can be obtained. Mixtures of fatty acid methyl esters were chromatographed on the adipate phase and the separations were excellent, but when aldehyde dimethyl acetals were chromatographed they reacted in some way with the adipate to produce a mixture of compounds which included unchanged aldehyde dimethyl acetal and some of the corresponding acid methyl ester! Fig. 5 shows a typical record of chromatographing a pure sample of myristaldehyde dimethyl acetal. The mechanism of this interaction is not understood but it may be acid catalysed because acetals are very susceptible to acid conditions.

The application of gas–liquid chromatography to mixtures of aldehydes isolated from natural sources has shown the very wide range of compounds present. Separations on a preparative scale should facilitate the isolation of unknown aldehydes and identification of their structures by chemical and physical methods.

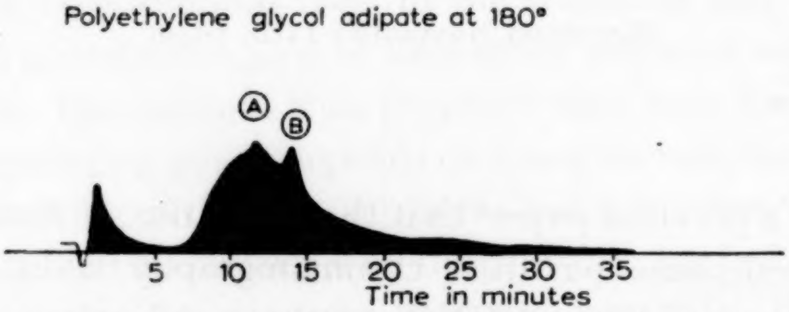

Fig. 5. Analysis of a pure sample of myristaldehyde dimethyl acetal on polyethylene glycol adipate stationary phase at 180°. Peak A shows unchanged myristaldehyde dimethyl acetal. Peak B shows presence of a second compound with retention volume identical to that of methyl myristate.

ACKNOWLEDGEMENTS

I should like to thank Dr. A. T. JAMES for his kind help and advice during the construction of the chromatographic apparatus and the British Empire Cancer Campaign for a grant for the apparatus.

SUMMARY

The long chain fatty aldehydes can be separated as their aldehyde dimethyl acetals by gas–liquid chromatography. Separations on Apiezon L grease and Reoplex 400 stationary phases are described.

The relative retention volumes of some pure aldehyde dimethyl acetals are given and compared with the relative retention volumes of the corresponding fatty acid methyl esters.

A tentative identification on the basis of their relative retention volumes on both stationary phases, is made of a number of unusual aldehydes found in mixtures isolated from natural sources.

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SEPARATION OF SOME RARE EARTHS BY REVERSED-PHASE PARTITION CHROMATOGRAPHY

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It has been shown in a previous paper[1] that the separation of zirconium from niobium by means of reversed-phase partition chromatography using tributyl phosphate (TBP) as the stationary phase and HNO_3 as the mobile phase is quite feasible. In the present paper an attempt to separate some of the rare earths by the same method is described. As the separation of rare earths is one of the most difficult problems of analytical chemistry and radiochemistry this attempt may be treated as a good test for the method and its future applications. From existing experimental data[2] it appears that such a separation should be possible, since the separation factor (the ratio of the two partition coefficients) is about 2 for neighbouring rare earths in the system concentrated HNO_3–TBP. This value is higher than the separation factor in adsorption on ion-exchange resins from citrate, glycolate, or lactate solutions[3]. The high separation factor together with the fact that the elution curves obtained by this method[1] were narrow, quite symmetrical, and without tailing promised a separation of neighbouring light rare earths. Some difficulties might be encountered with the heavier rare earths, since for these the separation factor is smaller and at low acid concentrations the order of the partition coefficients is reversed. In the present paper the possibility of separation of the lighter rare earths, terbium and yttrium has been described.

EXPERIMENTAL

Reagents

The following radioisotopes were used: ^{144}Ce, ^{147}Nd, ^{147}Pm, ^{153}Sm, $^{152,154}Eu$, ^{159}Gd, ^{160}Tb, ^{91}Y. ^{153}Sm, ^{159}Gd and ^{160}Tb were prepared by irradiation of Sm_2O_3, Gd_2O_3 and Tb_4O_7 in the Polish reactor "EWA" and the other isotopes were obtained from "Soyuzchim-export" (USSR).

TBP was purified by vacuum distillation and boiling with 5 % NaOH.

HNO_3 of AnalaR grade was used.

The kieselguhr "Hyflo Supercel" was first sieved and the fraction containing particles smaller than 0.08 mm was then mixed with water in a conical decanting flask. The portion that settled after about 10 minutes was collected, dried and treated with dichlorodimethylsilane as described by HOWARD AND MARTIN[4].

Preparation of columns and measurement technique

The following method for the preparation of columns was used: the column was filled with kieselguhr, previously treated as described above, and the bed slightly pressed with a glass rod after addition of each small portion of kieselguhr. As an alternative, uniform and compact packing of the column may be attained by shaking and tapping the column against some wooden support, after addition of each portion. An appropriate amount of TBP was then introduced and the column eluted with water under slight pressure to remove air. The time needed for the preparation of a column is about 2 h. The columns used in the course of this work were of about 3 mm diameter and contained 0.30 g of kieselguhr and 0.18 ml of TBP. The length of the bed was 11 cm. The columns thus prepared may vary somewhat in their separating power. The separating power depends on many factors, but the most important among them is the packing of the bed. When the bed is too loosely packed the elution curves are symmetrical but broad; when on the contrary, the bed has been too strongly pressed the elution curves are narrower but with long tails. Nevertheless it is not difficult to acquire sufficient skill in preparing columns that are equivalent to about 400 theoretical plates at 11 cm bed length and show only very slight tailing. The number of plates increases with the loading of the column when the diameter is kept constant. In the range from 0.3 to 0.6 mm, at constant bed length, the diameter itself has only a small influence. The column once prepared is stable and may be used for several months without changing its properties. Only when the concentration of HNO_3 is above 16 M are the changes more rapid, probably because of the dissolution of TBP. In the range from 0.2 ml/cm^2/sec to 0.8 ml/cm^2/sec the flow rate has only a small influence on the separating power of the column.

Separation procedure

0.05 ml of the active solution was introduced into the top of the column, which had been previously washed with HNO_3 of appropriate concentration saturated with TBP. The activity was then eluted under gentle pressure to achieve a flow rate of 0.4 ml/cm^2/sec or about 4 drops per min. Every fifth drop was collected, evaporated and its activity measured with an end-window Geiger-Müller counter. The free volume of the column was determined using ^{137}Cs.

RESULTS AND DISCUSSION

The possibility of achieving the following separations was investigated:

Ce–Nd–Pm. It is possible to separate these ions using 15.8 M HNO_3, although some overlapping of curves exists. See Fig. 1. Very good separation may be attained with 16.1 M HNO_3, but at this acid concentration columns are not stable.

Ce–Pm–Sm–Eu–Gd–Tb. This separation may be accomplished with 15.1 M HNO_3. As may be seen from Fig. 2 the separation is good, with small overlapping only in the case of the Eu and Gd curves.

Eu–Tb–Y. With 11.5 M HNO_3 Y is eluted after Tb and may be easily separated from it and all other light rare earths. Fig. 3.

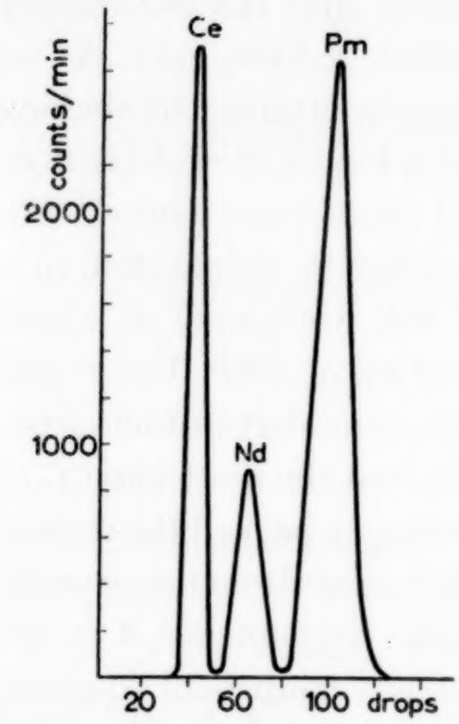

Fig. 1. Separation of Ce–Nd–Pm with 15.8 M HNO_3.

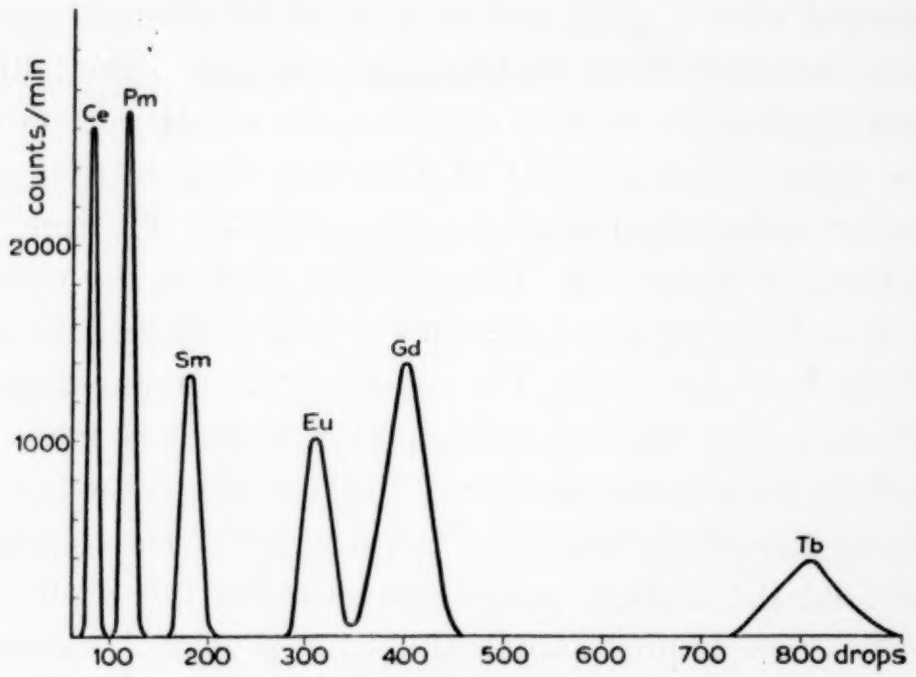

Fig. 2. Separation of Ce–Pm–Sm–Eu–Gd–Tb with 15.1 M HNO_3.

The possibility of the separation Ce–Pr–Nd was not investigated, but it seems that it would be difficult to accomplish it, since the partition coefficients for Ce and Pr are too small even in concentrated HNO_3.

In all the experiments described either carrier-free rare earths were used, or the quantities of rare earths introduced into the column were of the order of 0.02 mg (*e.g.* in the case of reactor irradiations). Some experiments were also carried out with larger quantities of rare earths. As a typical example the curve in Fig. 4 may be

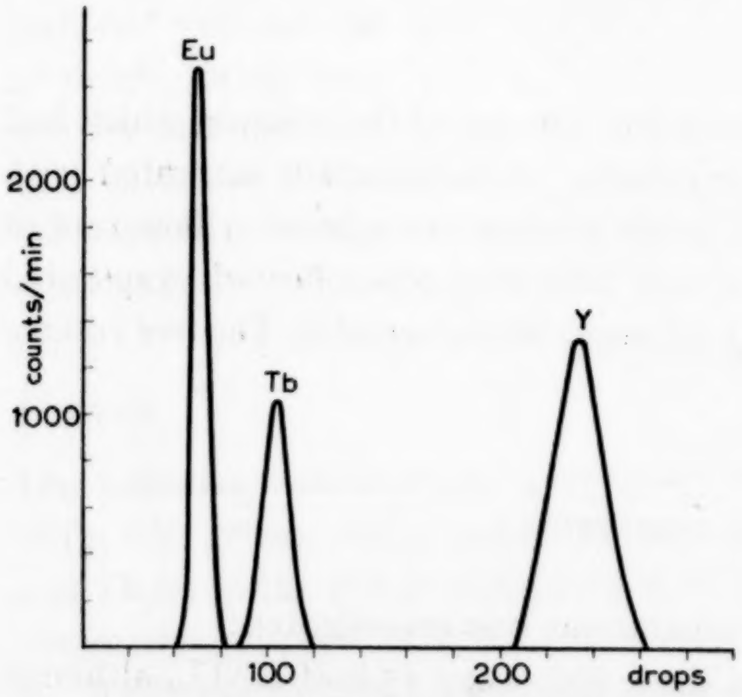

Fig. 3. Separation of Eu–Tb–Y with 11.5 M HNO_3.

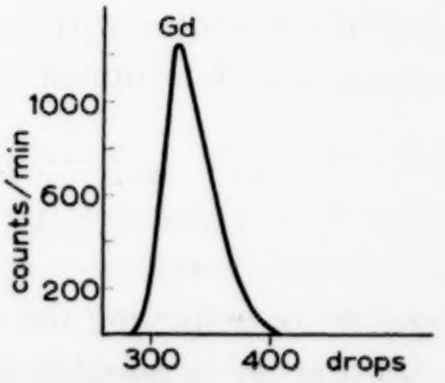

Fig. 4. Curve obtained with 0.5 mg Gd introduced into the column. Eluting agent, 15.1 M HNO_3.

quoted. It was obtained in an experiment with 0.5 mg of Gd introduced into the column. The curve with the front steeper than the rear is typical of an overloaded column. The position of the maximum is shifted towards the left in comparison with more dilute solutions of Gd (*cf.* Fig. 2).

The positions of the maxima of some earths when eluted with 15.1 *M* HNO_3 relative to the position of Eu are given in Table I. It follows from these data, that the separation factors for the Pm–Sm and Sm–Eu pairs are nearly equal and high, but that for the Eu–Gd pair the separation factor is exceptionally low. This behaviour is typical for rare earths since in the case of elution from ion-exchangers the Eu–Gd pair is also the most difficult to separate[5]. The separation factor for the Gd–Tb pair is higher than that for Eu–Gd and very near to the separation factors for Pm–Sm and Sm–Eu. Our data show that the half-filled shell effect in solutions of 15.1 *M* HNO_3 starts to operate between Eu–Gd rather than between Gd–Tb, and that because of the exceptional position of Eu–Gd the dependence of the partition coefficient on the atomic number cannot be exactly represented by two straight lines meeting at the position of Gd as was postulated for concentrated solutions of HNO_3 by PEPPARD and coworkers[2].

TABLE I

POSITIONS OF MAXIMA OF SOME RARE EARTHS RELATIVE TO THE POSITION OF EU

Pm	*Sm*	*Eu*	*Gd*	*Tb*
0.23	0.48	1.00	1.40	3.0

It follows from the above experiments that reversed-phase partition chromatography in the system TBP–HNO_3 may be successfully applied to the separation of small quantities of some rare earths. It is possible to separate the neighbouring rare earths from Pm to Tb, and with a change of the acid concentration from Ce to Tb, excluding Pr. It should be emphasized that the effluent from the column in this method does not contain any salts but only HNO_3 which is readily removed by evaporation and that the procedure is not time-consuming and may be satisfactorily carried out at room temperature.

SUMMARY

Reversed-phase partition chromatography has been applied to the separation of small amounts of some rare earths. Tributyl phosphate was used as the stationary phase, and the elution was carried out with concentrated HNO_3. The following separations were accomplished:

(1) Ce–Nd–Pm. Eluting agent, 15.8 *M* HNO_3;
(2) Ce–Pm–Sm–Eu–Gd–Tb. Eluting agent, 15.1 *M* HNO_3;
(3) Eu–Tb–Y. Eluting agent, 11.5 *M* HNO_3.

The method is rapid and may be carried out at room temperature.

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SELECTIVE SPRAY REAGENTS FOR THE IDENTIFICATION AND ESTIMATION OF FLAVONOID COMPOUNDS ASSOCIATED WITH CONDENSED TANNINS

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INTRODUCTION

Condensed tannins which are ubiquitously distributed in Nature, are in most instances accompanied by complex mixtures of flavonoid substances. Some of these function as important "precursors" in the biogenesis of the accompanying tannins, and their isolation is an essential prerequisite for satisfactory identification[1,2].

Paper chromatography furnishes a direct method of determining the complexity of the polyphenolic mixtures derived from barks and heartwoods, and usually assists in the prediction of the separation procedures which should be used for the effective resolution of components; for example, partition separations on cellulose or in the Craig machine or adsorption separations on cellulose. Furthermore, the identity of the compounds to be separated may often be established tentatively from a knowledge of the relation between R_F and the degree of hydroxylation and distribution of hydroxyl groups[3-5], coupled with the use of selective spraying reagents. Frequently it is also possible to estimate the concentration of flavonoid compounds in tannin mixtures by means of paper chromatography[6,7].

This paper discusses the accuracy with which chemical structure may be predicted through the use of five selective spray reagents, and describes the detail of a densitometric method of estimating flavonoid compounds based on the use of some of these reagents.

EXPERIMENTAL

Spray reagents

The following spray reagents have been found effective for the identification of flavan-3-ols (catechins), flavan-3,4-diols (leuco-anthocyanidins), flavonols, 2,3-dihydroflavonols, and may also be applied to flavanones, aurones and chalkones.

(*i*) *Ferric reagent.* 3 g AnalaR ammonium ferric sulphate (iron alum) in 100 ml water, used immediately after solution. The reagent gives sharp and distinct blues and greens with pyrogallol and catechol groups respectively. The ferric ion also coordinates weakly with mono- and *meta*-hydroxyphenols to give weak purple colorations observed only if the substances are present on chromatograms in excessively high concentrations.

TABLE I

COLOURS DEVELOPED BY SOME FLAVONOID COMPOUNDS AND THEIR CONSTITUENT PHENOLS WITH BIS-DIAZOTISED BENZIDINE

Compound	*Position of phenolic OH groups*	*Colour*
Phenols		
Phloroglucinol	1,3,5	deep purple
Resorcinol	1,3	claret-maroon
Phenol	1	very pale yellow*
Catechol	1,2	pale yellow**
Pyrogallol	1,2,3	pale reddish yellow**
Catechins or flavan-3-ols		
(+)-Gallocatechin and (—)-epimer	5,7,3′,4′,5′	claret-maroon
(—)-Epigallocatechin gallate	5,7,3′,4′,5′	claret-maroon
(+)-Catechin and (—)-epimer	5,7,3′,4′	claret-maroon
(—)-Epicatechin gallate	5,7,3′,4′	claret-maroon
(—)-Robinetinidol	7,3′,4′,5′	canary-yellow
(±)-Epifisetinidol	7,3′,4′	pale yellow
Leuco-anthocyanidins or flavan-3,4-diols		
Leuco-delphinidin	5,7,3′,4′,5′	claret-maroon
Leuco-cyanidin	5,7,3′,4′	claret-maroon
Leuco-robinetinidin	7,3′,4′,5′	canary-yellow
Leuco-fisetinidin	7,3′,4′	pale yellow
Leuco-guibourtinidin	7,4′	very pale yellow*
2,3-Dihydroflavonols or flavanonols		
Dihydromyricetin (Ampeloptin)***	5,7,3′,4′,5′	golden yellow
Dihydroquercetin (Taxifolin)	5,7,3′,4′	pale reddish yellow
Pinobanksin	5,7	red[11]
Strobobanksin	5,7 (6-methyl)	orange-yellow[11]
Dihydrorobinetin	7,3′,4′,5′	golden yellow
Dihydrofisetin (Fustin)	7,3′,4′	pale yellow
Flavanones		
Cryptostrobin	5,7 (8-methyl)	orange-yellow[10]
Strobopinin	5,7 (6-methyl)	yellow[10]
Pinocembrin	5,7	red[10]
Pinostrobin	5 (7-methoxy)	orange-red[10]
Flavonols		
Myricetin	5,7,3′,4′,5′	golden brown
Quercetin	5,7,3′,4′	reddish yellow
Robinetin	7,3′,4′,5′	golden brown
Fisetin	7,3′,4′	pale yellow
Flavones		
Chrysin	5,7	red
Tectochrysin	5 (7-methoxy)	very pale yellow*

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* Most colours develop instantaneously or over a few minutes but phenol, leuco-guibourtinidin and tectochrysin take periods of up to 10–15 min to develop.

** On aging catechol often turns pale brown and pyrogallol a brown yellow.

*** The name ampelopsin has been applied to both 3-β-glucosidopetunidin (WILLSTÄTTER AND ZOLLINGER[12]) and 5,7,3′,4′,5′-pentahydroxyflavanonol (KOTAKE AND KUBOTA[12]). The former has historical preference and the name ampeloptin was intended for the latter (personal communication from Dr. T. KUBOTA).

(*ii*) *Ammoniacal silver nitrate reagent.* This reagent due to PARTRIDGE[8] has been described by BATE-SMITH[3,9]. 14 g silver nitrate is dissolved in 100 ml water and 6 *N* ammonium hydroxide added until the silver oxide formed, just dissolves. In the cold the ammoniacal silver nitrate is instantly reduced by vicinal phenolic hydroxy groups, *e.g.*, *ortho*-dihydroxy and *ortho*-trihydroxy groups. Others, for example mono- and *meta*-hydroxyphenols reduce the ammoniacal silver nitrate very slowly. The shade of coloration developed, namely black, grey or brown, depends entirely on the concentration of the hydroxyphenol on the chromatogram and on the nature of the reducing group. Catechol, protocatechuic acid and some other catechol-containing units, if present in sufficiently high concentration give characteristic metallic grey colorations. Pyrogallol-containing units usually give black colorations if present in sufficiently high concentration and brown at low concentration. Catechol groups may give either black, grey or brown colours depending on concentration.

(*iii*) *Bis-diazotised benzidine.* This reagent due to LINDSTEDT[10] consists of two solutions: (a) Benzidine (5 g) or benzidine hydrochloride (6 g) stirred with conc. HCl (14 ml) and the suspension dissolved in water (980 ml). (b) 10% sodium nitrite. Two parts (b) are added to three parts (a) and the reagent used immediately after mixing. This reagent is one of the most selective available; the colours produced with flavonoid compounds and constituent phenols are given in Table I.

(*iv*) *Vanillin–toluene-p-sulphonic acid.* This reagent due to ROBERTS, CARTWRIGHT AND WOOD[4] is used by spraying a solution of vanillin (2 g) and toluene-*p*-sulphonic acid (1 g) in absolute ethanol (100 ml) and heating the chromatogram in an oven at 80–100° for 5–10 min.

"Flavonoid" substances containing phloroglucinol nuclei, *e.g.*, catechins, gallocatechins, leuco-cyanidin, leuco-delphinidin, dihydroquercetin, dihydromyricetin etc. give a strong violet-red colour, while those containing resorcinol nuclei, *e.g.*, robinetinidol, leuco-fisetinidin, fustin, etc., give weak pink colours after more prolonged heating.

(*v*) *Toluene-p-sulphonic acid.* This reagent due to ROUX[13] is a 3 % solution of toluene-*p*-sulphonic acid in absolute ethanol. After being sprayed lightly, chromatograms are heated at 80–100° for 5–10 min. Leuco-anthocyanidins, not hydroxylated in the 5 position, for example melacacidin, leuco-robinetinidin and leuco-fisetinidin give scarlet colorations. Leuco-anthocyanidins hydroxylated in the 5 position, for example leuco-delphinidin and leuco-cyanidin give yellow-red colours on more prolonged heating[5].

Estimation of flavonoid compounds by the maximum colour density method

For the estimation of flavonoid compounds in tannins the effective resolution of components on two-dimensional paper chromatograms is necessary. Improved resolution may be obtained by using larger sheets of paper so that the components are as widely separated from each other as possible. This assists the task of measuring the maximum colour densities of individual spots. The method followed is similar to that used by BLOCK[14,15] for the estimation of amino acids on two-dimensional chromatograms.

(*a*) *Apparatus*. Chromatograms are developed in two 20 in. × 20 in. × 8½ in. all-glass tanks (Shandon Scientific Co.) using upward migration in both directions. Micropipettes of 10 μl capacity ("Elphor", Bender and Hobein, Munich) are used for applying the substances, and even spraying is obtained with an all-glass spray-gun (Kopp Laboratory Supplies Inc., New York).

(*b*) *Paper*. Whatman No. 1 chromatographic paper cut into 16 in. squares are used. Spots of ½ in. diameter are applied at a point in one corner, 1 in. from each edge.

(*c*) *Preparation of standard catechin and tannin solutions*. 10 mg flavonoid compound is dissolved in 10 ml pure absolute ethanol. 10 μl quantities are applied on the spot of the above area until the desired concentration of the substance was obtained. Tannin solutions of 1 g per 10 ml are made up in ethanol or methanol and 10 μl applied on a spot of equal size.

(*d*) *Solvents*. For the first direction the upper phase of butan-1-ol–acetic acid–water (4:1:5) is used, and after drying in a strong current of air in a hood, the paper is irrigated with 2% acetic acid in water. Four chromatograms are run simultaneously. Development took place at 21° in a constant temperature room.

(*e*) *Time of development*. In the butanol–acetic acid–water mixture the chromatograms were run for approximately 20 h, and in 2% acetic acid for 8 h.

(*f*) *Development of colour and choice of spray reagents*. The papers may be sprayed with one of two reagents, the choice of reagent depending on the chemical nature of the flavonoid to be estimated. All chromatograms should be sprayed on both sides of the paper.

(i) Ammoniacal silver nitrate spray. In the best concentration range this reagent gives rather similar brown-black spots with compounds containing either pyrogallol or catechol nuclei. Where the choice of solvents and size of the chromatographic paper enables well-separated spots this reagent may be used to advantage, but it suffers from the disadvantage that it does not differentiate between substances containing differing groups. The use of dilute reagent must be avoided as the full intensity of the spot is not developed merely by heavy spraying. Immediately after spraying the paper sheets are washed three times with chloride-free water in a large (20 in. × 24 in.) stainless steel dish, then treated with 0.1% sodium thiosulphate, prepared from distilled water, for 3–4 min, and finally washed for 20 min in running tap water. All the washings are carried out in the dish and the chromatograms are allowed to air-dry in the dish to avoid tearing. Work in too high a concentration range should be avoided as the dense spots tend to "bleed" during washing.

(ii) Bis-diazotised benzidine spray. Only the freshly mixed solutions should be used. Flavonoid compounds containing a combination of resorcinol (A nucleus) and catechol (B nucleus) groups give very pale yellow colours usually unsuitable for measurement. Those containing resorcinol and pyrogallol (A and B nuclei) groups give deeper colorations ranging from canary-yellow to golden brown (see Table I) and may be estimated, although the silver nitrate reagent is preferred in both these instances. A combination of phloroglucinol (A nucleus) and catechol or pyrogallol (B nuclei) in catechins, *e.g.* (+)-catechin and (+)-gallocatechin, and flavan-3,4-

diols, *e.g.* leuco-delphinidin and leuco-cyanidin, gives an intense claret-maroon colour suitable for measurement. For estimating degradation-products obtained from flavonoid compounds the intense purple produced with phloroglucinol and the claret-maroon with resorcinol are also suitable.

Chromatograms sprayed with the reagent are allowed 1–3 min for full development of colours, and immediately washed for 20 min in running tap water to avoid a yellow back-ground due to excess benzidine reagent. The chromatograms are air-dried in a tray. The colours developed appear to be stable. Working in a high concentration range must be avoided due to the "bleeding" of intense spots during washing.

(*g*) *Measurement of colour density.* The maximum colour density of each spot is measured with a densitometer (Fig. 1) in which the intensity of the light source is controlled by a rheostat for coarse adjustment, and by an iris diaphragm for fine adjustment. The light intensity is measured with a selenium barrier layer photocell ("Eel" 37 × 50 mm) coupled directly with a sensitive galvanometer (Pye Scalamp Galvanometer 7891/S). The colour density of each spot is determined by using the blank paper of a particular chromatogram as 100 % transmission, and the average of replicate analyses at each concentration of flavonoid compound was plotted on semi-logarithmic paper. A straight-line relationship between log concentration and galvanometer reading was obtained for phloroglucinol (benzidine reagent), and for (—)-

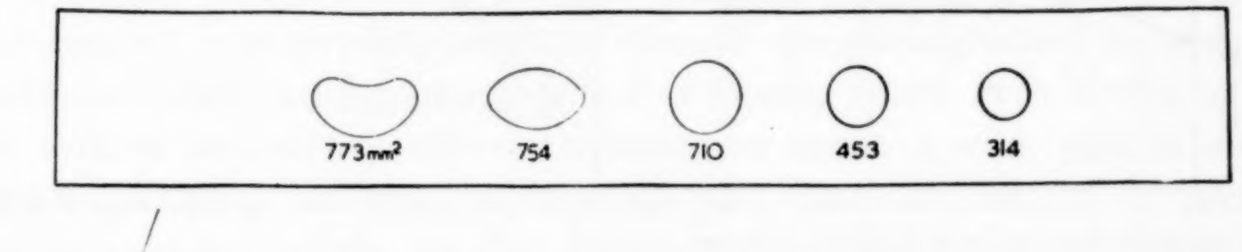

Fig. 1. Simple densitometer for measuring the maximum colour density on two-dimensional paper chromatograms. The sliding "mask" permits the use of different size apertures. One aperture only is selected for the preparation of a standard curve and for subsequent estimation.

robinetinidol, (+)-catechin and (+)-gallocatechin (benzidine and silver nitrate reagents) as in Fig. 2. Where compounds giving a pale yellow colour with the benzidine reagent, *e.g.* fisetinidol, fustin, leuco-fisetinidin and robinetinidol (at low concentration), are in close proximity or overlap the ochre colorations of (+)-catechin, (—)-epicatechin, (+)-gallocatechin, or epicatechin and epigallocatechin gallates, the maximum colour density of the latter may be determined by using a suitable green filter.

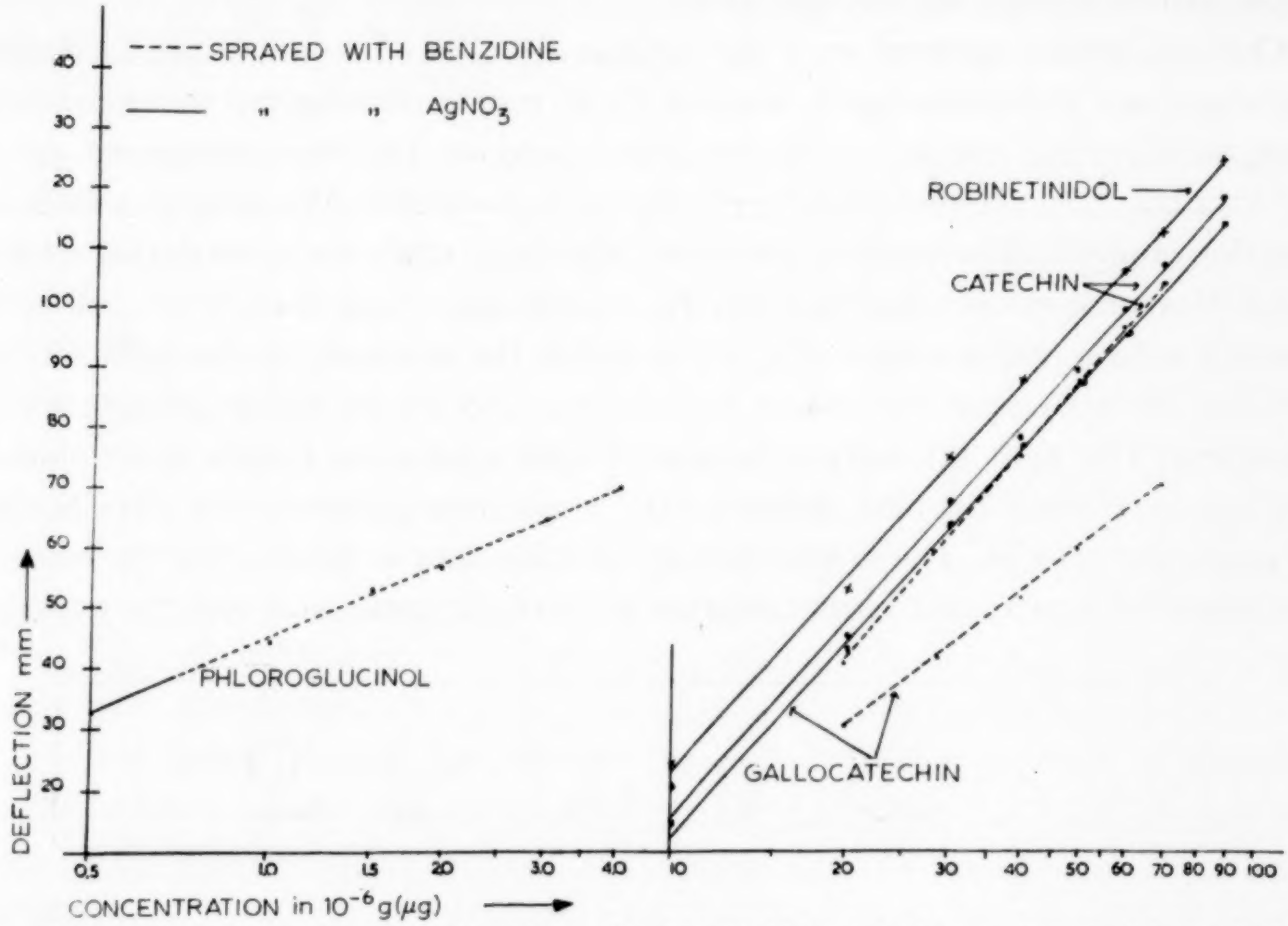

Fig. 2. Calibration curves for the estimation of robinetinidol, catechin, gallocatechin and phloroglucinol by the maximum density method.

Adsorption and partition chromatography of flavonoid compounds

(—)-Robinetinidol ((—)-7,3',4',5'-tetrahydroxyflavan-3-ol) has the same absolute configuration as (+)-catechin[16], and therefore also as (+)-gallocatechin[17]. The R_F values of (—)-robinetinidol in water-saturated *sec.*-butanol (0.67) and in water (0.42) were determined and compared with those of (+)-gallocatechin (0.54, 0.32)[5]. Similarly the R_F values of (±)-epifisetinidol ((±)-epi-7,3',4'-trihydroxyflavan-3-ol) (0.79, 0.42–0.47)[5] may be compared with that of (—)-epicatechin (0.60, 0.29). The naturally occurring enantiomorphous[24] (+)- and (—)-leuco-fisetinidins have the same R_F values in *n*-butanol–acetic acid–water (0.76) and in water-saturated *sec.*-butanol (0.68) but differ in their R_F values in water (0.54 and 0.48 respectively). The racemic mixture of these substances does not separate into the optical antipodes in water and runs to the lower R_F value (0.48). Conclusions drawn from these comparisons and from similar comparisons of the chromatographic behaviour of other flavonoid compounds of the resorcinol and phloroglucinol series[5], are described in the discussion section of this paper.

DISCUSSION

Selective spray reagents often furnish conclusive evidence regarding the A and B phenolic nuclei and the heterocyclic ring in flavonoid compounds.

The improved ferric reagent and the ammoniacal silver nitrate reagent afford information regarding the B nucleus when pyrogallol or catechol groups are present. When the B nucleus is monohydroxylated, for example the 4′-hydroxy groups in epiafzelichin, dihydrokaempferol and leuco-guibourtinidin, or unhydroxylated as in the flavones, flavanones and flavanonols typical of pine heartwood components[18], the state of hydroxylation of the nucleus may often be deduced from a knowledge of the hydroxylation of the A and heterocyclic rings coupled with the R_F of the substance in different solvent systems[5]. Other substances which present difficulties are dihydromorin (4′,6′-dihydroxy B nucleus), and melacacidin where both A and B nuclei have free catechol groups. Both compounds, however, readily undergo conversions into a flavonol and anthocyanidin respectively which may be identified.

The bis-diazotised benzidine and vanillin reagents afford information regarding the A nucleus when it consists of either phloroglucinol or resorcinol nuclei. The vanillin reagent is particularly sensitive towards the phloroglucinol nucleus giving a mauve-red colour after a short period of heating (often even in the cold) and almost without exception, while with resorcinol nuclei a weak pink is obtained with more prolonged heating. The bis-diazotised benzidine reagent is useful for confirming evidence of the vanillin reagent, giving claret-maroon colours with phloroglucinol-containing catechins and flavan-3,4-diols but not necessarily with phloroglucinol-containing 2,3-dihydroflavonols (see below). The benzidine reagent is of great diagnostic value and especially sensitive in its colour reactions, giving colours which are the sum-total of the colorations of the A and B phenolic moieties. It also reflects the presence of strong hydrogen bondage between 5-hydroxyl and 4-carbonyl groups when these are present. For example (*cf.* Table I), the resorcinol-containing flavonoids, fisetin, fustin, leuco-fisetinidin and fisetinidol give pale yellow colours derived mainly from the catechol B nucleus (yellow) and the monohydroxyphenol (very pale yellow) A nucleus. Robinetin, dihydrorobinetin, leuco-robinetinidin and robinetinidol give canary to deeper yellow colours (depending on concentration) derived mainly from the pyrogallol B nucleus which contributes a reddish yellow colour, and the monohydroxyphenol of the A nucleus contributing a very pale yellow. Where the A nucleus contains *meta*-hydroxy substituents, for example 5,7-dihydroxy groups, the deep claret-maroon colour developed is identical to that of the *meta*-dihydroxyphenol, resorcinol. This intense coloration presumably overshadows the pale yellow or deeper yellow of catechol and pyrogallol B nuclei. Typical examples are the catechins, gallocatechins and their gallates, leuco-cyanidins and leuco-delphindins.

The 2,3-dihydroflavonols belonging to the phloroglucinol series, for example dihydromyricetin (ampeloptin), dihydroquercetin (taxifolin) and dihydrokaempferol (aromadendrin) provide exceptions in that the colour produced with the bis-diazotised benzidine tends towards yellow and is either pale reddish yellow (dihydrokaempferol, dihydroquercetin) light golden brown (dihydromyricetin), orange-yellow (strobobanksin) or red (pinobanksin). Such behaviour probably reflects strong hydrogen bondage between the 5-hydroxyl and 4-carbonyl. This suggestion finds support in the finding that dihydrorobinetin and dihydromyricetin give completely identical light golden brown colorations with the benzidine reagent. The 2,3-dihydroflavonols of the phloroglucinol series nevertheless show the unmistakable presence of a phloroglucinol nucleus with the vanillin reagent.

Where the pattern of distribution of hydroxyl groups is "unusual", as for example in melacacidin (7,8,3′,4′-tetrahydroxyflavan-3,4-diol) the benzidine reagent behaves consistently in giving pale yellow colours, contributed presumably by "free" catechol groups of both A and B nuclei.

In flavonoid compounds some information regarding the heterocyclic ring is obtained by means of the toluene-*p*-sulphonic acid reagent. This sensitive reagent indicates the presence of 3,4-diol groups, by giving pink deep red colorations with flavan-3,4-diols unhydroxylated in the 5 position, for example, leuco-robinetinidin, leuco-fisetinidin and melacacidin[13]. Those flavan-3,4-diols which are hydroxylated in the 5 position, namely leuco-delphinidin and leuco-cyanidin give less characteristic orange colours[5]. The toluene-*p*-sulphonic acid reagent also converts 2,3-dihydroflavonols into flavonols during heating, and the emergence of fluorescence is usually detectable under ultra-violet light[13]. Other information regarding the oxidation state of the heterocyclic ring may be obtained under ultra-violet light where yellow fluorescence indicates either flavones or flavonols, the 5-deoxyflavonols giving exceptionally brilliant greenish-yellow fluorescence[3]. Anthocyanidins and anthocyanins show orange, pink, red and red-blue colorations with absorption-maxima lying in the range 500–555 mμ. Information supplied by the various spray reagents must in all instances be correlated with the R_F of the flavonoid compound in partitioning and adsorption solvent systems.

SWAIN AND HILLIS[19] reviewed methods of estimating total phenols, leucoanthocyanidins, flavonols in plant extracts and ROUX[20] used methods for estimating tannins (total polyphenols) in plant extracts. These methods are applied to heterogeneous mixtures of compounds, and they are of necessity empirical. KING AND WHITE[21] have studied a number of methods for estimating individual groupings in tannins or phenolic compounds. The estimation of individual "flavonoid" compounds in mixtures derived from natural sources has not apparently been attempted.

The application of paper chromatography and the elution procedures commonly used for sugars, presents difficulties as the recovery of flavonoid substances from the cellulose is never quantitative. Substantial losses occur apparently due to the residual affinity of flavonoid compounds for cellulose, the losses varying according to the chemical nature of the compound. These difficulties are overcome by the use of two-dimensional paper chromatography coupled with the measurement of maximum colour density similar to that used by BLOCK[14, 15] for the estimation of amino acids and amines. Various catechins and phloroglucinol give a straight line relationship between galvanometer deflection and log concentration using ammoniacal silver nitrate and bis-diazotised benzidine reagents. The choice of spray reagent for estimation depends on the ability of the compound either to reduce strongly or to couple strongly with the reagent. The benzidine reagent is more versatile in that phloroglucinol-containing flavan-3-ols and flavan-3,4-diols, affording a claret-maroon colour, may be estimated in the presence of resorcinol-containing flavonoid compounds, which give various shades of yellow, with the aid of a suitable green filter. By means of these methods catechins have been estimated in tannin mixtures[6], and catechins and their degradation products in the presence of tannins formed by the autoxidation of catechin[7]. This densitometric method is of obvious value in the micro-degradation of flavonoid compounds and tannins.

In previous work[5] dealing with the correlation between structure and chromatographic behaviour of flavonoid compounds comparison of flavan-3-ols (catechins) and 2,3-dihydroflavonols (flavanonols) of the resorcinol series with those of the phloroglucinol series was overlooked. The flavan-3-ols, (—)-robinetinidol and (±)-epifisetinidol have higher R_F values in water (0.42, 0.42–0.47) than their corresponding 5-hydroxy derivatives, (+)-gallocatechin and (—)-epicatechin (0.32, 0.29), of identical absolute configuration[16,17]. A similar effect is evident (see Table I of ref. [5]) in the comparison of the R_F values in water of the naturally-occurring dihydroflavonols, dihydrorobinetin (0.35) and ampeloptin (0.24), fustin (0.36) and taxifolin (0.28), all of which are likely to have the 2,3-*trans* configuration of substituent groups in the heterocyclic ring[22]. The presence of a hydroxyl group in the 5 position, therefore, causes a marked decrease of the R_F in water or in 2–6 % aqueous acetic acid, even in the 2,3-dihydroflavonols of the phloroglucinol series (ampeloptin and taxifolin) where hydrogen bondage between 5-hydroxyl and 4-carbonyl occurs[23].

In partitioning solvents the naturally-occurring enantiomorphous (+)- and (—)-leuco-fisetinidins run to the same R_F (0.68 in *sec.*-butanol) but their R_F values in water differ (0.54, 0.48 respectively) as is the case for catechin, gallocatechin and their epimers[25]. Unlike these catechins, however, the racemic mixture of (+)- and (—)-leuco-fisetinidins does not separate in water due probably to molecular interaction, although their R_F values differ markedly when run individually.

Hydrogenation of the 2,3 double bond in the heterocyclic ring in both the pairs flavone → flavanone[10] and flavonol → flavanonol[5] increases the R_F in partitioning mixtures[10,5].

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SUMMARY

Five selective spray reagents provide information regarding the A and B phenolic nuclei and the heterocyclic ring of flavonoid compounds. This information coupled

with R_F values in two solvent systems, enables the tentative identification of a wide variety of flavonoid substances. LINDSTEDT's bis-diazotised benzidine reagent gives colour reactions which are highly indicative of variations in the distribution of phenolic hydroxyl groups. The benzidine and ammoniacal silver nitrate reagents may be used with two-dimensional paper chromatography and a simple densitometer for estimating flavonoid compounds present in tannins and also the yield of phenols resulting from the degradation of flavonoid compounds and tannins. The benzidine reagent allows for the estimation of flavan-3-ols and flavan-3,4-diols of the phloroglucinol series in the presence of their counterparts of the resorcinol series.

Additional factors in the correlation between structure and chromatographic behaviour of flavonoid compounds are described.

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IDENTIFIZIERUNG ORGANISCHER VERBINDUNGEN

XXXVI. MITTEILUNG. PAPIERCHROMATOGRAPHISCHE TRENNUNG UND IDENTIFIZIERUNG VON AMINOANTHRACHINONEN*

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In der Literatur wurden bereits einige Methoden zur papierchromatographischen Trennung von Aminoanthrachinonderivaten und der von diesen Verbindungen abgeleiteten Dispersionsfarbstoffen vorgeschlagen. MITCHELL[1] chromatographierte solche Farbstoffe auf mit Sojabohnenöl vorbehandelten Papieren unter Benützung des wässrigen Methylcellosolves als bewegliche Phase, ZAHN[2] trennte einige Cellitonfarbstoffe mit Hilfe von 60 % Aethylenglykol; ELLIOT UND TELESZ[3] mit Petroläther gesättigt mit Methanol und MICHEEL UND SCHWEPPE[4], sowie JANOUŠEK[5] empfielen auf dem azetylierten Papier zu chromatographieren. FRANC[6] chromatographierte einige Mono- und Diaminoanthrachinone in Systemen mit Dimethylformamid, Formamid, Azetamid und Harnstoff als stationären Phasen und vor kurzem beschrieb CASTIGLIONI[7] die Trennung von 1- und 2-Aminoanthrachinon im System Benzol–Essigsäure und Benzol–Salzsäure.

In dieser Mitteilung wollen wir eine Methode beschreiben, die wir seit einigen Jahren in unserem Laboratorium zur Trennung, Identifizierung und Reinheitsprüfungen von Aminoanthrachinonderivaten benützen. Wir chromatographieren entweder auf umgekehrten Phasen (1-Bromnaphthalin/Pyridin + Wasser) oder auf mit Formamid imprägnierten Papieren und Toluol oder Chloroform als Fliessmittel.

EXPERIMENTELLER TEIL

Alle Versuche führten wir auf dem Whatman papier No. 3 durch. Zur Imprägnierung wurden die Papierstreifen durch eine 10 % methanolische 1-Bromnaphthalinlösung oder eine 20 % äthanolische Formamidlösung durchgezogen und dann durch Aufhängen auf der Luft 15 Minuten lang bei Zimmertemperatur getrocknet. Auf in dieser Weise vorbehandelte Papiere wurden die chromatographierten Substanzen in Form von ½ % Pyridinlösungen (1–2 μl) aufgetragen. Chromatographiert wurde absteigend, die Temperatur schwank im Bereich von 19 bis 22°. Zur Chromatographie auf mit 1-Bromnaphthalin vorbehandelten Papieren benutzten wir als bewegliche Phase Mischungen von Pyridin und Wasser im Verhältnis 1:1 oder 2:1. In beiden Fällen

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TABELLE I

R_F-WERTE DER AMINOANTHRACHINONDERIVATE

No.	Anthrachinon*	R_F-Werte im System**				Farbe der Flecken
		A	B	C	D	
I	1-Amino-	0.38	0.56	0.92	0.96	rotorange
II	1-Amino-2-methyl-	0.25	0.47	0.96	0.96	rotorange
III	1-Amino-4-chlor-	0.25	0.46	0.93	0.95	rotorange
IV	1-Amino-5-chlor-	0.25	0.48	0.95	0.96	rotorange
V	1-Amino-2-methyl-4-brom-	0.13	0.32	0.97	0.97	rotorange
VI	1-Amino-2,4-dibrom-	0.06	0.19	0.97	0.97	rotorange
VII	2-Amino-	0.60	0.72	0.62	0.86	gelb
VIII	2-Amino-3-chlor-	0.30	0.50	0.86	0.96	gelb
IX	2-Amino-1,3-dibrom-	0.13	0.30	0.97	0.97	gelb
X	1,2-Diamino-	0.70	0.80	0.28	0.70	rotviolett
XI	1,4-Diamino-	0.74	0.81	0.51	0.81	violett
XII	1,5-Diamino-	0.55	0.70	0.76	0.90	rot
XIII	1-Oxy-4-amino-	0.33	0.52	0.92	0.96	rotviolett
XIV	1-Amino-2-brom-4-oxy-	0.06	0.16	0.98	0.98	rotviolett
XV	1-Oxy-4-amino-8-chlor-	0.20	0.45	0.93	0.96	rotviolett
XVI	1,4-Diamino-2,3-dichlor-	0.10	0.27	0.96	0.98	blauviolett
XVII	2,3-Diamino-	0.30	0.50	0.87	0.93	gelb
XVIII	2,6-Diamino-	0.89	0.94	0.04	0.19	***
XIX	2,7-Diamino-	0.89	0.94	0.04	0.19	braunorange
XX	2-Amino-3-oxy-	0.78	0.79	0.10	0.32	gelbbraun
XXI	1-Phenylamino-	0.04	0.17	0.97	0.97	rot
XXII	1-Benzoylamino-	0.03	0.15	0.97	0.98	gelb
XXIII	1-Benzoylamino-4-amino	0.10	0.27	0.96	0.97	violettrot
XXIV	1,4-Dibenzoylamino-	0.01	0.07	0.98	0.98	rot
XXV	1-Benzoylamino-5-chlor-	0.02	0.12	0.98	0.98	gelb
XXVI	1-Benzoylamino-4-methoxy-5-chlor-	0.06	0.20	0.97	0.98	rot
XXVII	Alizarintürkisblau B (Base) C.I. 61525	0.03	0.14	0.98	0.98	blaugrün
XXVIII	Alizarinbrillantgrün Extra B (Base) C.I. 61565	0.02	0.04	0.98	0.98	blaugrün
XXIX	Alizarinbrillantgrün 5G (Base)	0.02	0.03	0.98	0.98	hellgrün
XXX	Alizarinviolanol R (Base) C.I. 60725	0.02	0.09	0.98	0.98	violettblau
XXXI	Alizarinblauschwarz B (Base) C.I. 63615, Sch. 1195	1.00	1.00	0.00	0.00	violett
XXXII	Alizarinblau RF (Base) C.I. 62080	0.07	0.19	0.98	0.98	blau
XXXIII	Alizarinrosanol R (Base) C.I. 68210, Sch. 1210	0.11	0.27	0.91	0.98	karminrot
XXXIV	4,4′-Diamino-1,1′-dianthrimid C.I. 65005	0.19	0.45	0.84	0.96	graublau
XXXV	Alizarinreinblau B (Base) C.I. 62100, Sch. 1199	0.02	0.13	0.98	0.98	blau
XXXVI	Alizarinechtgrau 2G	0.01	0.02	0.98	0.98	grüngrau
XXXVII	Toluidinblau (Base) C.I. 63340	0.01	0.03	0.98	0.98	blau

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[*] Bei den Basen der sauren Anthrachinonfarbstoffe werden die Nummern der entsprechenden Farbstoffe nach *Colour Index*[8] oder *Farbstofftabellen*[9] angegeben.

[**] A = 1-Bromnaphthalin/Pyridin–Wasser (1:1); B = 1-Bromnaphthalin/Pyridin–Wasser (2:1); C = Formamid/Toluol; D = Formamid/Chloroform.

[***] Nach Besprühen mit einer *p*-Dimethylaminobenzaldehydlösung intensiv braunoranger Flecken.

musste die bewegliche Phase mit 1-Bromnaphthalin gesättigt werden, um das Auswaschen der stationären Phase während der Chromatographie zu vermeiden. Im Falle des Formamids als stationäre Phase benutzten wir Toluol oder Chloroform als bewegliche Phase.

Alle chromatographierten Verbindungen wanderten auf den Chromatogrammen als intensiv gefärbte Flecken, nur zur Sichtbarmachung des 2,6-Diaminoanthrachinons mussten wir von der Nachweisreaktion mit einer *p*-Dimethylaminobenzaldehydlösung (1 g *p*-Dimethylaminobenzaldehyd gelöst in 95 ml Aethanol und 5 ml konz. Salzsäure) Gebrauch machen.

Die R_F-Werte, sowie die Farbe der Flecken sind in der Tabelle I angeführt.

RESULTATE UND DISKUSSION

Die Resultate unserer Versuche zeigten, dass das geeignetste Lösungsmittelsystem zur papierchromatographischen Trennung der Aminoanthrachinonderivate 1-Bromnaphthalin/Pyridin–Wasser im Verhältnis 1:1 oder 2:1 ist. In diesem System bilden alle geprüften Derivate scharf abgegrenzte, runde Flecken; die Trennung ist scharf und es können befriedigend bis 60 μg Stoff chromatographiert werden. Die Methode ist weiter auch sehr einfach und ist aus allen diesen Gründen den Methoden früherer Autoren weit überlegen. Die R_F-Werte schwanken zwar etwas mit der Temperatur, die gute Trennung wird jedoch nie beeinflusst. Mit zunehmendem Gehalt an Pyridin in der beweglichen Phase wachsen die R_F-Werte an, durch Zugabe von Wasser werden sie vermindert. Bei Benützung des Lösungsmittelgemisches Pyridin–Wasser im Verhältnis 1:1 werden die Mono- und Diaminoderivate gut voneinander getrennt, die Basen der sauren Anthrachinonfarbstoffe bleiben am Start; ist das Verhältnis 2:1, wandern die unsubstituierten Diaminoanthrachinone in der Nähe der Lösungsmittelfront, die Monoaminoderivate werden dagegen sehr gut voneinander getrennt und in diesem System wandern auch schon die Basen der sauren Farbstoffe.

Für die Reinheitsprüfungen von technischem 2,6-Diaminoanthrachinon benützten wir mit Erfolg weitere zwei Lösungsmittelsysteme und zwar Papier imprägniert mit Formamid und Toluol oder Chloroform als bewegliche Phase. 2,6-Diaminoanthrachinon hat nämlich in diesen Systemen den kleinsten R_F-Wert — es verbleibt praktisch am Start — und alle in Frage kommenden Verunreinigungen wandern auf dem Chromatogram und werden voneinander gut getrennt. Ein weiterer Vorteil dieser Systeme mit Formamid als stationäre Phase ist die Schnelligkeit; das Lösungsmittel durchlauft die Strecke von 40 cm vom Start in zwei Stunden. Dagegen haben diese Systeme den Nachteil dass viele von den geprüften Stoffen zur Bildung von Schwänzen neigen, sodass die Trennungen nicht mehr so scharf sind auch in den Fällen, wo die R_F-Unterschiede grösser sind als auf mit 1-Bromnaphthalin imprägnierten Papieren. Auch die Menge der chromatographierten Stoffe ist beschränkt, und viele von den Aminoanthrachinonderivaten wandern mit der Lösungsmittelfront und werden nicht getrennt. Mit Hilfe dieser Methoden fanden wir im Rückstand nach der Extraktion von technischem 2,6-Diaminoanthrachinon mit Azeton folgende 8 Verunreinigungen:

im System Formamid/Toluol mit R_F-Wert 0.00; 0.04; 0.10; 0.13; 0.17; 0.28; 0.62; 0.92 und im System Formamid/Chloroform 0.06; 0.19; 0.31; 0.49; 0.54; 0.70; 0.86 und 0.96. Von diesen konnten wir bisher 1- und 2-Aminoanthrachinon und 1,2- und 2,7-Diaminoanthrachinon identifizieren.

CHEMISCHE STRUKTUR UND CHROMATOGRAPHISCHES VERHALTEN

Für das Verhalten der Aminoderivate des Anthrachinons auf den Papierchromatogrammen lassen sich auf Grund unserer Resultate, die in der Tabelle I zusammengefasst sind, einige Gesetzmässigkeiten ableiten und zum Teil begründen. In Folgendem sollen die Resultate auf den mit 1-Bromnaphthalin vorbehandelten Papieren diskutiert werden. In diesen Systemen ist der R_F-Wert zunächst von der Anzahl der freien Aminogruppen abhängig: die Diaminoderivate besitzen grössere R_F-Werte als Monoaminoderivate. Durch Benzoylierung der freien Aminogruppe oder durch Arylierung dieser wird der R_F-Wert wieder stark herabgesetzt (siehe z.B. I – XXII; XI – XXIII – XXIV; IV – XXV; I – XXI; XI – XXVIII). Die Verminderung der Polarität der chromatographierten Verbindungen verschiebt also die Löslichkeit dieser Stoffe zu Gunsten der weniger polaren stationären Phase. Eine Herabsetzung der R_F-Werte wird weiter durch Substitution der Anthrachinone durch Methylgruppe oder durch Halogenatome bewirkt (siehe z.B. I – II; I – III – IV – VI; XXII – XXV; VII – IX; XI – XVI). Bemerkenswert ist das Verhalten der Alizarinblauschwarz B-Base (XXXI). Man kann es durch Strukturverschiedenheit von den anderen chromatographierten Verbindungen erklären. Es wurde schon früher gezeigt, dass in dieser Verbindung ein 1-Oxy-2 (oder 3), 10-dianilido-4,9-anthrachinon und kein 9,10-Anthrachinonderivat vorliegt[10].

Auf den Papieren, die mit Formamid imprägniert sind, ist natürlich das Verhalten der Verbindungen umgekehrt.

DANK

Frau O. Beranová danke ich für Durchführung eines Teils der experimentellen Arbeit.

ZUSAMMENFASSUNG

Es wird eine Methode zur papierchromatographischen Trennung und Identifizierung von Aminoanthrachinonen auf mit 1-Bromnaphthalin vorbehandelten Papieren und Gemischen von Pyridin und Wasser als mobilen Phasen beschrieben. Zur Reinheitsprüfung von 2,6-Diaminoanthrachinon bewährten sich die Systeme Formamid/Toluol oder Formamid/Chloroform.

SUMMARY

A method is described for paper-chromatographic separation and identification of aminoanthraquinones on paper impregnated with 1-bromonaphthalene, using

mixtures of pyridine and water as mobile phases. For the purity control of 2,6-diaminoanthraquinone the systems formamide/toluene or formamide/chloroform proved advantageous.

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A MODIFIED TWO-DIMENSIONAL PAPER CHROMATOGRAPHIC SYSTEM FOR THE SEPARATION OF DNP-AMINO ACIDS

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The 2,4-dinitrophenyl (DNP) derivatives of amino acids are used most often in studies on the determination of the structure of proteins and peptides. BISERTE, *et al.*[1] have recently reviewed the methods of paper chromatography for the separation of these amino acid derivatives. The two-dimensional paper chromatographic system employing a "toluene" solvent and 1.5 *M* phosphate buffer[2] is presently considered the most satisfactory for the separation of ether-soluble DNP-amino acids normally found in proteins. The "toluene" solvent, which is a mixture of toluene, pyridine, chloroethanol and ammonia, has been criticized on two points. It is not convenient to use this solvent in laboratories where either nitrogen or colorimetric ninhydrin determinations are being carried out routinely. Further, the two-phase "toluene" system requires several hours of equilibration before use and then the organic phase, used for the development of the chromatogram, is not stable. Other two-dimensional paper chromatographic systems which have been proposed have only substituted a different organic solvent for the "toluene" solvent[3,4].

In the solvent systems presently described, the phthalate buffer proposed by BLACKBURN AND LOWTHER[5] is used. The paper (Whatman No. 1, 22 in. × 18 in.) is dipped in 0.05 *M* phthalate buffer* at pH 6.0 and then air dried at room temperature. The solution of DNP-amino acids** is placed in one corner of the paper and the chromatogram equilibrated with an atmosphere of *n*-amyl alcohol and water for 2 hours. The chromatogram is then developed in a descending fashion for 15–18 hours with *n*-amyl alcohol saturated with 0.05 *M* phthalate buffer. The paper is allowed to air dry in the dark at room temperature. This usually requires about 3 to 6 hours. The chromatogram is then developed in the second direction for about 15 to 18 hours using 1.5 *M* sodium sulfate–0.05 *M* phthalate buffer at pH 6.0, in a descending direction. Usually it is necessary to allow the aqueous solvent to drip from the end of the paper chromatogram in order to have the amino acid derivatives move far enough for good resolution. The *n*-amyl alcohol–phthalate buffer solvent can be stored for at least six months, if kept in an air-tight container.

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* The 0.05 *M* phthalate buffer was prepared by mixing 50 ml of 0.1 *M* potassium acid phthalate and 45.45 ml of 0.1 *N* NaOH and diluting to 100 ml with CO_2-free water[5].

** All the DNP-amino acids used in this study were prepared by the method of SANGER[6] or that of LEVY AND CHUNG[7].

The movement of 19 DNP-amino acids and 2,4-dinitrophenylaniline in both solvent systems, in relation to the movement of 2,4-dinitrophenol, is given in Table I. The distances moved were measured from the point of origin to the center of the spot. Dinitrophenol moves about 20 cm from the origin after development for 17 hours with *n*-amyl alcohol–phthalate buffer solvent system at 23–25° (R_F = 0.5–0.55). In the 1.5 M sodium sulfate–phthalate buffer solvent system, dinitrophenol moves about 12 cm from the origin in 17 hours at 23–25° (R_F = 0.2–0.25).

TABLE I

RELATIVE MOVEMENT OF DNP-AMINO ACIDS ON PAPER CHROMATOGRAMS

DNP-amino acid	*n-Amyl alcohol–phthalate buffer*	*Sulfate–phthalate buffer*
Aspartic acid	0.03	2.09
Glutamic acid	0.09	1.90
Cystine (di-DNP)	0.10	0.24
Serine	0.16	1.65
Histidine (di-DNP)	0.21	0.15
Glycine	0.22	0.98
Threonine	0.38	1.80
Alanine	0.58	1.26
Ornithine (di-DNP)	0.61	0.13
Proline	0.68	1.59
Lysine (di-DNP)	0.92	0.13
Methionine	0.98	1.03
Dinitrophenol	1.00	1.00
Arginine	1.02	1.02
Tyrosine (di-DNP)	1.10	0
Tryptophan	1.17	0.27
Valine	1.25	1.33
Phenylalanine	1.27	0.60
Isoleucine	1.43	1.35
Leucine	1.43	1.35
Dinitroaniline	1.66	0.42

The difference in the relative positions of the DNP-amino acids on the chromatograms between the presently described two-dimensional system and the systems previously reported is due mainly to the organic solvent used in the first dimension. The sulfate–phthalate buffer system gives a separation of the DNP-amino acids which is essentially the same as that obtained with the 1.5 M phosphate buffer. The sulfate–phthalate buffer solvent system was found to give more distinct spots with sharper boundaries for most of the DNP-amino acids than when the phosphate buffer solvent system was used, especially when the amino acid derivatives had first been separated with the use of the *n*-amyl alcohol–phthalate buffer system.

The general distribution of the DNP-amino acids on the chromatogram developed with *n*-amyl alcohol–phthalate buffer closely resembles the distribution obtained with butan-2-ol–0.05 M phthalate buffer at pH 6.0[5]. The R_F of the DNP-amino acids which move more slowly than dinitrophenol is less when the *n*-amyl alcohol solvent is used than when the butan-2-ol solvent is used. Furthermore, the DNP-derivatives of glycine, serine, threonine and alanine can be completely resolved on a unidimensional chromatogram with the *n*-amyl alcohol–phthalate buffer system.

The presently described solvent systems cannot be used to separate DNP-methionine, α-DNP-arginine and dinitrophenol. However, α-DNP-arginine is not found in the DNP-amino acids which are extractable from aqueous media with ether; dinitrophenol can be removed by sublimation or by bleaching with HCl fumes.

SUMMARY

The two-dimensional separation of DNP-amino acids by solvent systems which appear to overcome most of the shortcomings of previously described solvent systems is described. The organic solvent consists of *n*-amyl alcohol saturated with 0.05 *M* phthalate buffer at pH 6.0; the aqueous solvent consists of 1.5 *M* sodium sulfate containing 0.05 *M* phthalate buffer (pH = 6.0).

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Short Communications

Chromatography on paper impregnated with zirconium phosphate

During the last few years numerous authors have carried out chromatographic separations of various ions by using paper of which the natural exchange capacity had been increased by introducing suitable functional groups into the cellulose molecule[1-6], and also by using paper impregnated with ion-exchange resins[7-11]. In the latter case, the possible separations depend above all on the type of resin used and not on the type of paper employed.

The advantages of paper containing ion-exchange groups as compared with the usual chromatographic papers or ion-exchange columns, were mentioned by TUCKERMAN[4], LEDERER[8] and HALE[11].

In this paper a separation method on filter paper impregnated with synthetic inorganic ion exchangers is described.

Zirconium phosphate (ZP) was chosen as inorganic ion exchanger, because its ion-exchange capacity can be compared with that of ion-exchange resins, and because it might show a different selectivity for different ions[12-13].

Several attempts were made to prepare strips by dipping them into a freshly prepared suspension, as used by LEDERER[7,8] for the preparation of paper impregnated with ion-exchange resins, but so far without success. We were, however, able to precipitate ZP directly onto the paper fibres.

Preparation of the ion-exchange paper

Strips (6 × 40 cm) of Whatman No. 1 filter paper were drawn as uniformly as possible through a 30% solution of $ZrOCl_2$ in 4 *N* HCl. After this impregnation the strips were quickly placed, for 5 min, on pieces of filter paper, which immediately absorb the excess liquid. The impregnated paper was then dried at room temperature by placing it on another sheet of filter paper. The dry strips were then dipped for 2 min into a 60% solution of H_3PO_4 in 4 *N* HCl, and dried at room temperature. After 6 h the excess H_3PO_4 was removed from the strips by washing them first in 2 *N* HCl for 10 min and then twice in H_2O.

In order to increase the ZP exchange capacity[14], an additional treatment with the same 60% solution of H_3PO_4 in 4 *N* HCl was carried out and afterwards the strips were put into an oven at 50° for 75 min.

As described above, the strips were subsequently washed with 2 *N* HCl, H_2O and left to dry in the air.

It is important to note that special chromatographic paper is not required for the preparation of these strips. Ordinary filter paper can be used because the R_F value depends essentially on the type of ion exchange employed. In any case all that is required to obtain the same results is to employ the same type of paper and above all to standardize the preparation of the sheets as far as possible, so that the same quantity of ions per unit area is obtained.

Experimental

The solutions of metal ions were prepared by dissolving the chlorides.

The chromatogram (Whatman No. 1 filter paper impregnated with 3.5 mg/cm^2 of ZP) was 40 cm long and the starting point was situated at 3.5 cm from one end. The strip was developed by the ascending method, with HCl of various concentrations, for 4–8 hours. In Table I the R_F values obtained are given. The values reported show that it is possible to carry out several separations by employing this eluent.

On studying the behaviour of alkaline earths and alkali metals it was found that the same order of adsorption is obtained on ZP-impregnated paper as on ZP columns.

TABLE I

R_F VALUES IN HCl OF VARIOUS CONCENTRATIONS

*Element**	R_F		
	0.1 N HCl	*0.5 N HCl*	*1 N HCl*
Hg(II)	0.67	—	—
Cd(II)	0.60	—	—
Cu(II)	0.59	—	—
Bi(III)	—	—	0.90
Fe(III)	0	0	0.04
Al(III)	0.13	0.40	0.90
**Cr(III)	0.75	0.80	—
Mn(II)	0.60	0.80	—
Ce(III)	0.19	—	—
La(III)	0.31	0.73	0.80
Ti(IV)	0	0	0
UO_2(VI)	0	—	—
Th(IV)	0	0	0
Co(II)	0.67	—	—
Ni(II)	0.61	0.80	0.85
**Zn(II)	0.69	—	—
**Ca(II)	0.81	—	—
**Sr(II)	0.81	—	—
Ba(II)	0.60	—	—
***Ra(II)	0.44	—	—
Mg(II)	0.76	—	—
Li(I)	0.82	—	—
Na(I)	0.68	0.77	0.83
K(I)	0.53	—	—
Rb(I)	0.12	—	—
**Cs(I)	0	0	—

* The spots were detected by spraying the strips with suitable reagents.
** Starred elements were also detected by radiochemical methods.
*** Only radiochemical methods were employed for radium.

To achieve further separations, which were not possible with HCl, mixtures of NH_4Cl–HCl, NaCl–HCl and KCl–HCl at various concentrations and ratios were used. In Fig. 1 a few representative separations are shown.

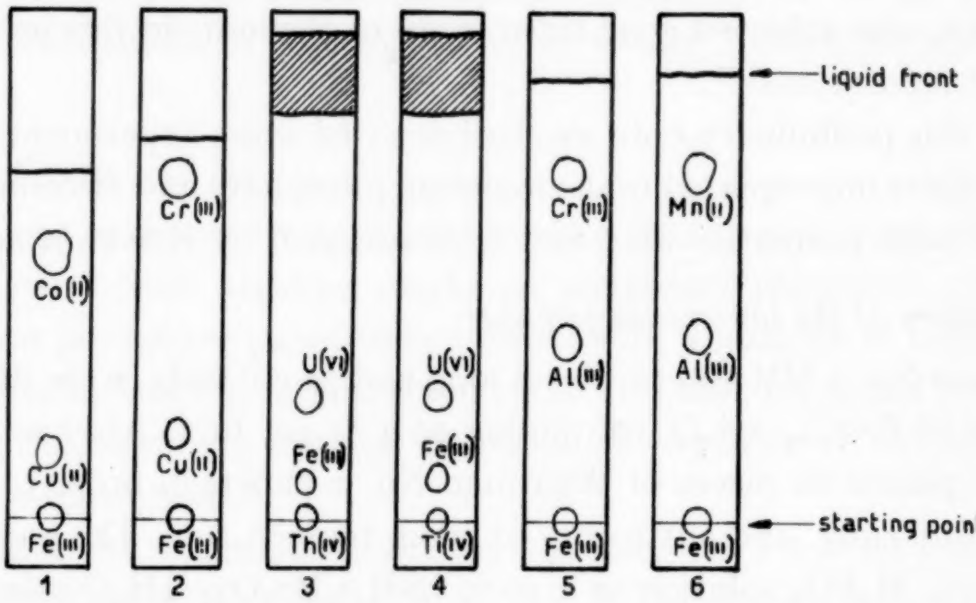

Fig. 1. Representative separations on ZP-paper using: (1) and (2) 0.035 N HCl and 0.045 M NaCl (1:1); (3) and (4) 2 N HCl and 4 M NH_4Cl (1:1); (5) and (6) 0.5 N HCl. The shaded area represents the localization of several cations, amongst which were La, Ce, Ni, Co, Cr and Al.

The results obtained indicate that ZP-impregnated paper may thus be successfully employed for the chromatographic separation of numerous cations.

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Chromatography on paper impregnated with inorganic ion-exchangers

The impregnation of filter paper with ion-exchange resins has been carried out by LEDERER, who achieved good separations of elements in this way and predicted other possible separations[1-3].

In this preliminary note we shall describe some experiments, of the same nature, using papers impregnated with zirconium phosphate and zirconium molybdate, whose ion-exchange properties were first demonstrated by KRAUS *et al.*[4,5].

Preparation of the ion-exchange paper

Whatman No. 3 MM sheets, 28 cm long and 21 cm wide, were dipped into an aqueous solution of $ZrOCl_2 \cdot 8H_2O$, containing 20 g Zr per liter. After impregnation they were quickly placed on pieces of Whatman No. 1 paper, in order to absorb the excess of liquid uniformly, and left to dry at room temperature. The papers were then dipped into a 3% H_3PO_4 solution or a 10% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ solution, transferred to a dish with distilled water, again placed on pieces of Whatman No. 1 paper and left to dry. Finally the edges of the sheets were cut off to eliminate errors due to a possible accumulation of the exchanger on those regions.

The treated paper has the appearance of untreated paper, but it is a little more hardened.

Separation of Ca, Sr and Ba

We tried to repeat on paper the work of KRAUS *et al.* concerning the separation of alkaline earths on zirconium molybdate columns.

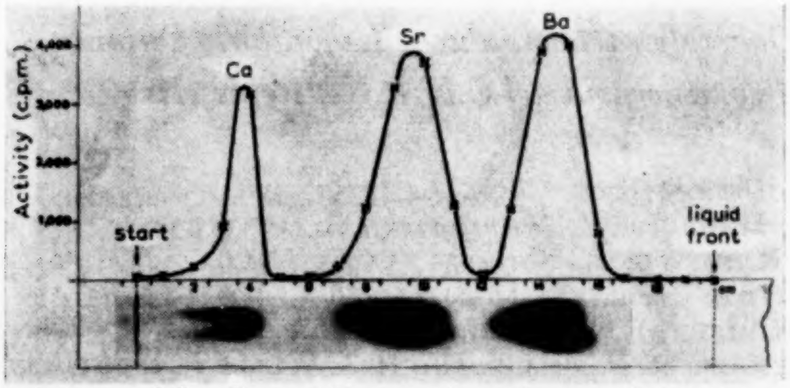

Fig. 1. Separation of a mixture of Ca, Sr and Ba on zirconium molybdate paper, with 0.9 N NH_4Cl for ~ 1h 40 min.

Strips of zirconium molybdate paper 25 cm long and 3 cm wide were spotted with a mixture of ^{45}Ca, ^{89}Sr, ^{140}Ba in 0.5 N HCl and developed for 20 cm with a NH_4Cl solution by the ascending method. The two last-mentioned radionuclides were in the carrier-free state.

To establish the most convenient concentration of the eluent, development was carried out with NH_4Cl solutions of various concentrations, ranging from 0.1 to 1.3 N. The best separation was obtained with 0.9 N NH_4Cl; this is shown in Fig. 1. It should be noted that the curve and the autoradiogram of this figure represent the radioactivity of the chromatogram measured 1 week after separation. If the measurements were made shortly after elution another maximum was observed situated in the starting region before Ba, due to ^{140}La ($T\frac{1}{2}$ = 40 h), which was in radioactive equilibrium with ^{140}Ba.

Experiments were also performed with a mixture containing 5 μg of each element, traced with the same radionuclides. The separation is similar though the R_F values are slightly higher.

Separation of Cs from Ca, Sr and Ba

We also made an attempt to repeat on paper the work of KRAUS *et al.* concerning the separation of alkali metal from alkaline earths on zirconium phosphate columns.

Strips of zirconium phosphate paper were spotted with a mixture of carrier-free ^{137}Cs and of the radionuclides of Ca, Sr and Ba in 0.5 N HCl and developed for 15 cm with HCl solution by the ascending method. Development was carried out with HCl solutions of various concentrations.

It was observed that the separation is already possible with 0.1 N HCl and good results are obtained with a wide concentration range of the eluent. Fig. 2 shows the results obtained with 0.5 N HCl.

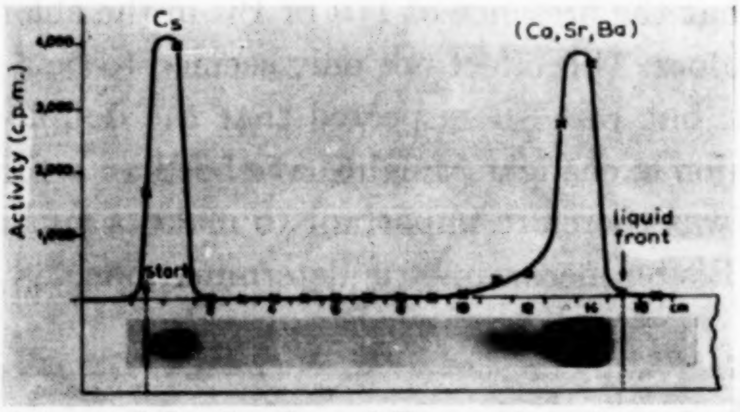

Fig. 2. Separation of a mixture of Cs, Ca, Sr and Ba on zirconium phosphate paper, with 0.5 N HCl for ~ 45 min.

In another series of experiments a salt form (NH_4^+) of the exchanger was used. Strips of zirconium phosphate paper were dipped into a N NH_4OH solution, washed with distilled water and left to dry at room temperature. They were then spotted with the same mixture and developed with a 2 N NH_4Cl solution.

A good separation was also obtained, but in this case the position of the elements is inverted, which agrees essentially with the results of KRAUS *et al.*

The separation is equally good for quantities of the order of 5 μg of each element.

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The influence of tris(hydroxymethyl)aminomethane and of monomethyl bis(hydroxymethyl)aminomethane on mercurimetric chloride determinations

The properties of tris(hydroxymethyl)aminomethane (Tris) and monomethyl bis(hydroxymethyl)aminomethane (Bis) buffers have made them very useful in biochemical work[1,2]. These compounds are stable, very soluble in water, and do not give precipitates with calcium ions. Their inhibitory action is in most cases low, and so is their extinction coefficient at 280 mμ. It has also been a special advantage to have such cation buffers available for anion exchange chromatography of proteins[3] in the pH range from 7 to 9. As it is highly desirable to determine the concentration of the exchanging ion, Tris–HCl or Bis–HCl buffers have often been chosen. Chloride concentration may easily be determined in a large number of fractions by the mercurimetric method[3,4].

However, when studying chloride adsorption isotherms on diethylaminoethyl cellulose[5], we found that the presence of Tris or Bis in the chloride solutions gave too high mercurimetric values. This effect not only seemed to be a serious source of error in the isotherm work, but we also suspected that the double-fronting phenomenon observed earlier on anion exchangers[6] might have been an artifact due to a high concentration of Tris. It was therefore important to make a quantitative study of the influence of Tris and Bis on mercurimetric determinations.

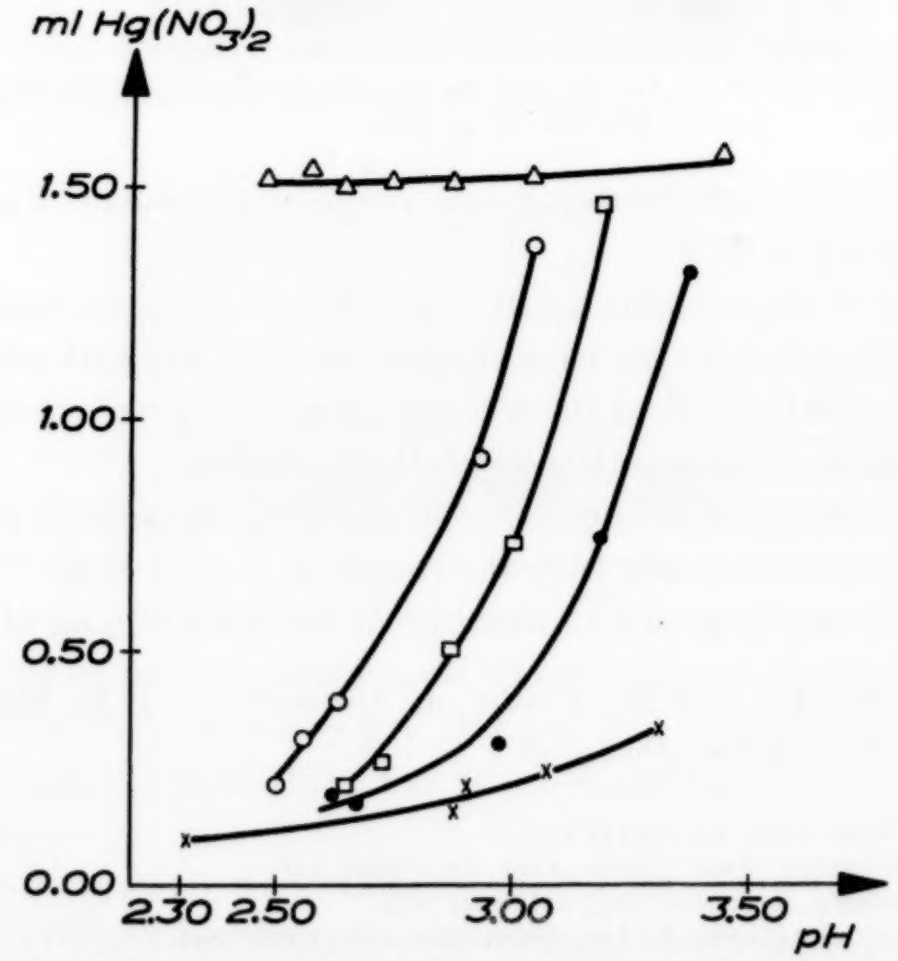

Fig. 1. (a) Titrations of 1.0 ml 0.01 *M* sodium chloride (△—△) and of various amounts of 0.8 *M* Tris, 0.5 ml (×—×), 1.0 ml (●—●), 1.5 ml (□—□) and 2.0 ml (○—○), with 0.007 *N* $Hg(NO_3)_2$ at different end-point pH values. Sulfuric acid and sodium hydroxide were used for adjusting the pH. The blank value for water was 0.10 ml $Hg(NO_3)_2$. Temp. 23–24°.

The Tris and Bis used were Commercial Solvents Corporation products, but the very pure Sigma Tris product "Sigma 121" showed the same behaviour. All the experiments were made with freshly prepared solutions. Mercuric nitrate and sodium nitroprusside was obtained from The British Drug Houses Ltd.

Fig. 1a shows the results of mercurimetric titrations at different end-point pH values. The nearly horizontal curve represents sodium chloride without Tris, and the other curves different amounts of Tris without any chloride present. At a pH higher than about 2.3, Tris alone will remove mercuric ions from the solution, and this effect increases strongly with increasing pH. It can also be seen that the amount of mercuric nitrate required is not proportional to the Tris concentration at a fixed pH. This deviation from linearity is more pronounced at higher pH values.

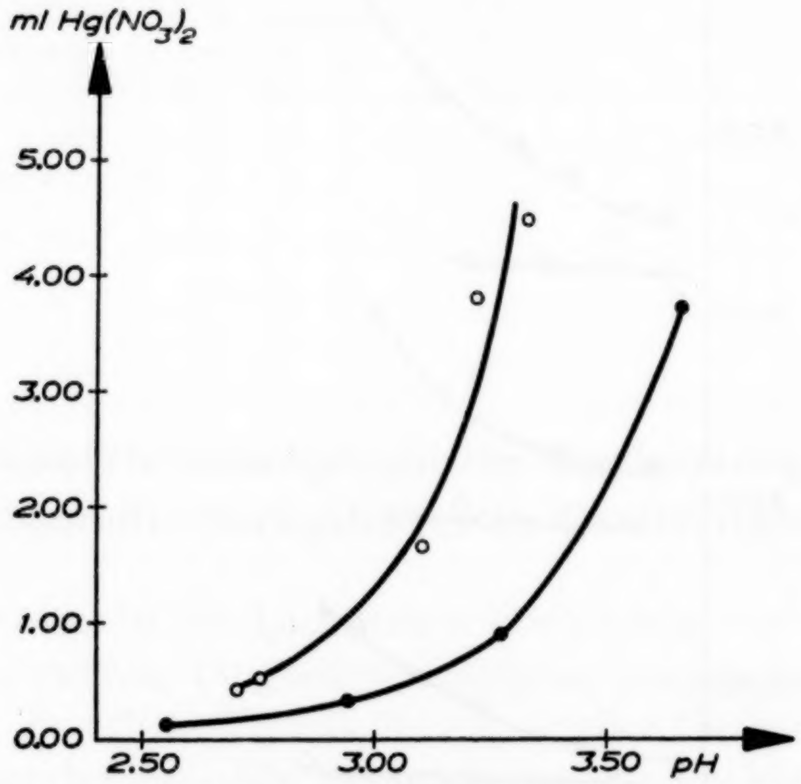

Fig. 1 (b) Titrations of 2.0 ml (●——●) and 4.0 ml (○——○) 0.8 *M* Bis under the same conditions as in Fig. 1 (a).

The Bis influence is pH-dependent in a similar way (Fig. 1b), but a larger amount of Bis is required to produce the same effect. The 2 ml Bis curve is practically coincident with the 1 ml Tris curve, and the 4 ml Bis curve is very close to the 2 ml Tris curve.

It seems natural to conclude that mercuric ions form a complex with Tris or Bis, but this cannot be the whole truth. When a solution of Tris–HCl is titrated, the amount of mercuric nitrate consumed is larger than what would be expected from the separate complexing actions of Tris and chloride. If the Tris concentration and the end-point pH are fixed, a higher chloride concentration gives a larger difference between the Tris–HCl curve and the NaCl curve, as we have shown in Fig. 2. This influence is stronger at higher pH values and may be proportional to the chloride concentration (subtraction of the 1 ml Tris curve in Fig. 1a from the Tris–HCl curves in Fig. 2 does not reveal a strict linearity, but the discrepancies may be within the limits of the experimental error).

Different hypotheses for the underlying mechanism have been considered, but at present we have no evidence for any of them. It is possible that Tris may act as a reducing agent, and B. G. MALMSTRÖM has in fact observed (personal communication, *cf.* also [7]) a reduction of ferrous ion by a Tris solution.

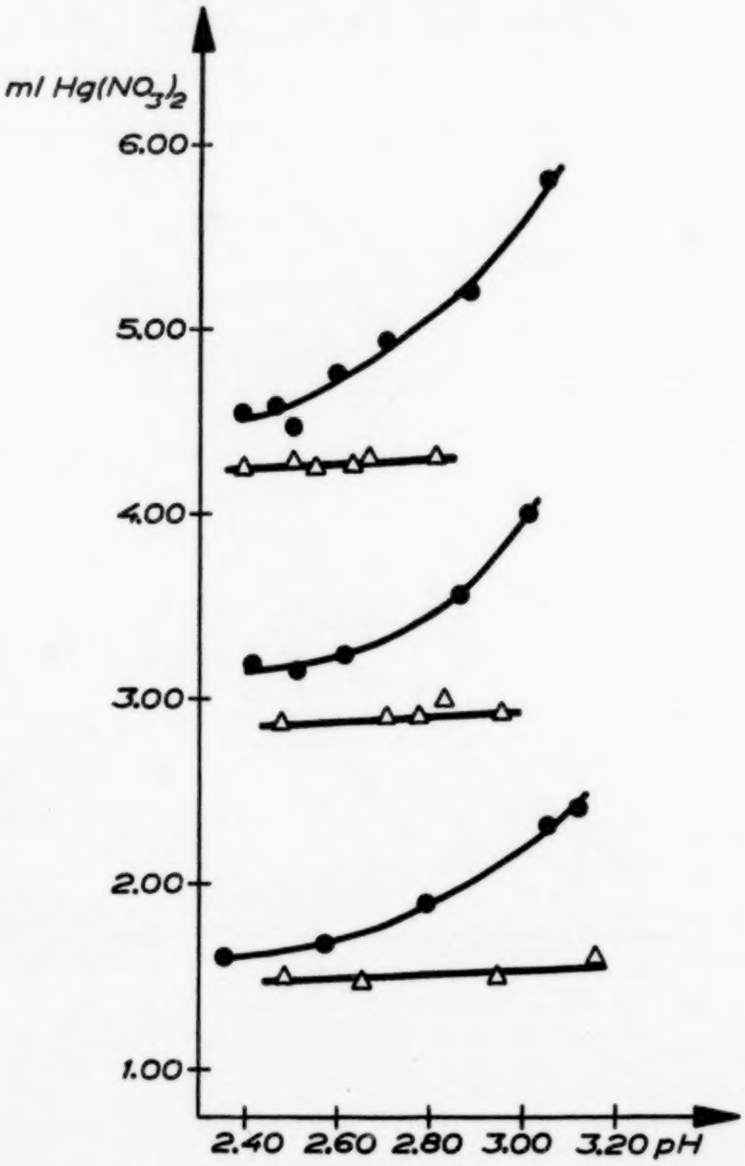

Fig. 2. Titrations of 1.0 ml, 2.0 ml and 3.0 ml 0.01 M sodium chloride without Tris (△——△), and with 1.0 ml 0.8 M Tris added (●——●). Other conditions the same as in Fig. 1 (a).

Obviously, the determinations of chloride in Tris–HCl buffers will in some cases be seriously disturbed by the above-mentioned influences. This is especially true for buffers in the upper part of the buffer range, where the total Tris concentration is much higher than the chloride concentration. Tris buffers containing several anionic species, *e.g.* chloride, carbonate and borate together, have been used by some authors, and the Tris–chloride ratio will then be high even near the lower limit of the buffer range. To avoid these disturbances, the end-point pH in the titration should be kept low, preferably at about 2 or lower. Very accurate determinations can be made by first ashing the sample together with an excess of sodium hydroxide, and then titrating as usual. In this way we have ascertained that the double-fronting of chloride in anion-exchange chromatography[6] is not an artifact.

The Tris–chloride–mercuric ion interactions may also provide a basis for a method of determining Tris concentration. This work is now being continued.

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J. Chromatog., 4 (1960) 88–91

Papierchromatographische Bestimmung des Gibberellinsäuregehaltes in Gärflüssigkeiten

Es wird eine Methode für die quantitative Bestimmung von Gibberellinsäure als Produkt der metabolischen Tätigkeit verschiedener Stämme von *Fusarium moniliforme* beschrieben, die sich der Technik der absteigenden Chromatographie im System Butylacetat–Wasser bedient. Alle Bestimmungen werden unter Verwendung der üblichen Papiersorten (Whatman No. 1 oder Schleicher-Schüll No. 598) auf Grund der Technik des Überfliessens bei sägeförmig zugeschnittenem unterem Papierrande durchgeführt. Auf jeden Papierstreifen werden 12 Proben und zwar sechs Standardproben (2 × 20 μg, 2 × 60 μg, 2 × 120 μg) und sechs Gärflüssigkeitsproben aufgetragen.

Für Standardpräparate wird krystallinisches Gibberellinsäurepulver verwendet, das in einer 0.1 M KH_2PO_4-Lösung aufgelöst wird, deren pH mittels 2 N HCl auf 2.5 bis 3.0 eingestellt worden ist. Endkonzentrationen zu 20, 60 und 120 μg/ml werden durch Verdünnung einer Vorratslösung von 1000 μg/ml mittels KH_2PO_4-Lösung hergestellt. In eingeschliffenen Zentrifugier-Röhrchen werden je 5 ml der hergestellten Standardlösung zusammen mit je einem ml *n*-Butanol geschüttelt, bis der Wirkungsstoff in die organische Phase übergeht. Sodann werden jeweils 0.05 ml dieser organischen Phase auf die Startpunkte der chromatographischen Papierstreifen aufgetragen (Durchmesser der Flecke 0.7 bis 0.8 cm), deren Gibberellinsäuregehalt dem eines ml der Gärflüssigkeit äquivalent gehalten werden soll.

Die Gärflüssigkeitsproben entsprechen 5.5 bis 6 ml des Kulturfiltrats. Nach der Einstellung des pH mittels 2 N HCl auf 2.5 bis 3.0 werden die ausgeflockten Unreinigkeiten abzentrifugiert und 5 ml Supernatans werden mit je einem ml *n*-Butanol ähnlich wie die Standardproben geschüttelt. Sodann werden ebenfalls wieder 0.05 ml der organischen Phase auf das chromatographische Papier aufgetragen. Sie bringen Ergebnisse in μg/ml.

Nach der wie üblich vorgenommenen Einlage des Papiers in den chromatographischen Zylinder dauert der Atmosphärenausgleich in demselben und die Sättigung des Papiers 10 bis 18 Stunden. Zum Schlusse wird das Chromatogramm 7 Stunden lang entwickelt.

Die Gibberellinsäure wird nach der Beendung des Chromatographieprozesses nach der Methode von OVERBEEK *et al.*[1] sichtbar gemacht. Ihr Gehalt wird sodann in Werten der Flächengrösse des Probeflecks von einer Kalibrationskurve abgelesen, die aus Standardwerten (120, 60 und 20 μg) konstruiert ist.

Die Anwendung des Systems Butylacetat–Wasser erscheint für die quantitative Bestimmung eines Stoffes mit einer Karboxylgruppe von der Art der Gibberellinsäure zweckmässig. Durch Dissoziationseinfluss entstehen bei der Chromatographie in diesem neutralen System langgezogene Flecke und man gewinnt so eine ausreichende Abstufung der Fleckgrösse der untersuchten Proben mit einem verschiedenen Gehalt an Gibberellinsäure.

Die besten Ergebnisse erzielt man bei Flächengrössen, die 40 bis 100 μg Gibberellinsäure entsprechen. Deshalb soll die Probe mittels KH_2PO_4-Lösung annähernd auf diese Konzentration eingestellt werden. Das Maximum der Fehlerquelle dieser Bestimmung beträgt etwa 10 bis 15%. Die untere Grenze der Empfindlichkeit der Methode liegt bei 5 μg.

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ERRATUM

J. Chromatog., 3 (1960) 353–356.

Bei den Figuren 1–7 muss es heissen: "Stationäre Phase: Di-*n*-decylphthalat (Säule A)" und nicht "Stationäre Phase: Siliconöl (Säule C)".

CENTRIFUGALLY ACCELERATED PAPER CHROMATOGRAPHY OF CHLOROPLAST PIGMENTS*

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(Received October 26th, 1959)

The techniques of centrifugally accelerated paper chromatography were developed by McDonald *et al.* in 1957[1]. The combination of two vectors, chromatography and centrifugal acceleration, makes it possible to separate compounds in very short times, for examples in 15 minutes instead of 8 hours. The chlorophylls and carotenoids are very labile compounds which undergo marked decomposition in the lengthy paper chromatography necessary for adequate resolution. It was hoped that this difficulty might be overcome by the use of rapid centrifugally accelerated paper chromatography.

The literature contains separations of water-soluble compounds, such as dyes and amino acids[2], but as yet there is no mention of separation of any other compounds. Therefore, a study was made of the fundamental factors involved in obtaining good separations and reproducible chromatograms of the algal pigments with nonpolar solvents.

EXPERIMENTAL

The centrifugally accelerated chromatograms were made on the chromatofuge which was assembled and operated in the prescribed manner**. All the experiments reported were carried out at 25°. Whatman Nos. 1 and 3 MM circles were used, as well as circles cut from the very thick Whatman No. 17 MM sheets. Since most of the pigments were required for spectroscopic and radioactive measurements or for further chromatography, it was desirable to use the thicker Whatman Nos. 3 MM or 17 MM papers. The pigment extract was placed on a circular origin which must be at least 8 cm in diameter. It was most satisfactory to apply the pigments in ethereal solution, as the ether evaporated quickly; however, other solvents were also feasible. With practice it was possible to make very thin uniform circles of pigment on the origin by holding the paper vertically with one hand and applying the extract with the other; papers were spotted in the cold room with as little light as possible.

The total algal extract which could be placed on such a circular origin on a Whatman No. 3 MM sheet was equivalent to 0.3 to 0.4 mg pigment, *i.e.* the extract obtained from 0.05 ml wet packed algal cells. About three times as much material could be placed on the circular origin of a Whatman No. 17 MM paper. Since the paper was thick it was necessary to spot the material on *both* sides or else the pigment zones on the underside of the paper after development, would have lower R_F values.

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* The work described in this paper was sponsored by the United States Atomic Energy Commission, University of California, Berkeley, Calif.

** Labline No. 5060 Chromatofuge, Labline Inc., Chicago, Ill.

It was also desirable to use a liner for the chromatofuge head. A sheet of Whatman No. 3 MM paper was placed in close contact with the bottom of the head, by cutting holes in the sheet so that the stainless steel points protruded through the liner paper. Some of the solvent was placed on this liner and the machine rotated for 5 min in order to saturate the chamber head with the solvent fumes, before placing the paper to be developed in the chromatofuge. In subsequent runs, about 10 to 20 ml of solvent was poured on to the liner, the paper placed in position and developed. It was not necessary, in the subsequent runs, to rotate the machine for the 5-min period, before the development of the paper.

In the case of petroleum ether–isopropanol solvents, it was necessary to mix the solvent several days before it was required for use. Fresh solvent mixtures used under the same running conditions gave anomalous results (the solvent did not travel nearly as far and the separations obtained were very poor).

RESULTS AND DISCUSSION

First of all it should be pointed out that the chromatograms obtained, despite the velocity of the rotation, are not circular in shape but elliptical, as was reported by McDONALD *et al.*[2] for aqueous solvents. This is not a disadvantage however, since measurement of R_F values from different parts of the ellipse gave values which were within the experimental error ($\pm$ 0.02 R_F units).

It was found that the resolution of the carotenoids and chlorophylls was the same as that obtained in conventional ascending or descending chromatography; the latter methods have been reviewed by ŠESTÁK[3]. That is, any solvent which gave satisfactory resolution in the conventional methods could be used for the centrifugally accelerated chromatograms, although it was necessary to determine suitable running conditions for the chromatofuge.

Most of the factors reported by McDONALD *et al.*[2] for development of the chromatograms with aqueous solvent, were found to be effective in these experiments with nonpolar solvents. In order to obtain reproducible R_F's it is necessary to have a flow rate of solvent such that the perimeter of the wetted area does not increase after the rotor and solvent flow are stopped. It is also necessary to avoid such a high flow rate that flooding of the paper will occur during development, as this causes distortion of the zones.

The placement of the circular origin is important; it must not be less than 7.5 cm in diameter or else some of the pigments will migrate inwards. It was found that an increase of 1.0 cm for the diameter of the circular origin resulted in an increase of about 0.04 for intermediate R_F values. It was not advantageous to increase the diameter beyond 12 cm since under those conditions only spots with R_F values less than 0.3 would be separated.

In general, under the same conditions of nitrogen pressure, velocity of rotation, and with the same delivery jet, different solvents will travel different distances, as might be expected. For example, at 500 r.p.m., 7.5 p.s.i. nitrogen and a flow rate of 2.0 ml/min, toluene traveled 15 cm; petroleum ether (b.p. 75°), 13 cm; petroleum ether (b.p. 30–60°), 6 cm; and petroleum ether–isopropanol (100:2.5) 14 cm in 10 min.

Also with different grades of paper, the distance traveled by the solvent will vary. With a flow rate of 2.5 ml/min of toluene at 500 r.p.m. the results given in Table I were obtained.

TABLE I

	Time of development (min)	*Origin → solvent front (cm)*
Whatman No. 1	10	16
	12.5	17.5
	15	19
Whatman No. 3 MM	10	14
	12.5	15
Whatman No. 17 MM	10	6
	20	14
	25	16

It can be seen that increasing the time results in a nonlinear extension of the solvent front. Obviously when trying out a new grade of paper or a different solvent, it is necessary to make some trial runs with no pigments on the paper to find a satisfactory time.

Yet another variable needs to be considered: the use of the liner paper. This is essential with most solvents, in particular with petroleum ether solvents. Even with the heavier Whatman Nos. 3 MM or 17 MM circles, it is necessary to have a liner.

Table II illustrates the R_F values obtained, using the same solvent and conditions, at different velocities. In general, it is seen that above 450 r.p.m., the R_F decreases as

TABLE II

CHROMATOFUGE R_F VALUES OF ALGAL PIGMENTS

Conditions: Whatman No. 3 MM paper (unwashed). Solvent petroleum ether–isopropanol (100:2.5); flow rate of 2.5 ml/min; 7.5 p.s.i. nitrogen pressure; temp. 25°; liner saturated with 20 ml of solvent; time of development 10 min; circular origin 10 cm. The R_F values reported are the average of 3 separate runs at each velocity quoted.

Speed in r.p.m.	*350*	*400*	*450*	*500*	*550*	*600*	*650*	*750*	*850*	*950*
Carotenes	0.91	0.92	0.94	0.94	0.96	0.95	0.97	0.96	0.97	0.98
Lutein	0.46	0.47	0.56	0.47	0.44	0.42	0.36	0.35	0.32	0.30
Chlorophyll *a*	0.44	0.45	0.47	0.38	0.37	0.30	0.26	0.27	0.23	0.22
Chlorophyll *b*	0.27	0.26	0.26	0.23	0.23	0.16	0.14	0.14	0.11	0.08
Neoxanthin	0.06	0.08	0.10	0.13	0.11	0.11	0.08	0.05	0.05	0.04

Note: As in ascending or descending chromatograms, with petroleum ether–isopropanol (100:2.5), violaxanthin is masked in the bottom of the chlorophyll *a* spot.

the velocity of rotation increases, although for a change of 50 r.p.m. the decrease in the R_F is within the experimental error. Changes however, obtained over the complete range of velocities available (*i.e.* 350 to 950 r.p.m.) are significant. It will be noticed that between 350 and 450 r.p.m. the R_F values remain the same or increase with increasing velocity, while the general pattern in the range 450 to 950 r.p.m. is one of decreasing R_F values. The speed at which the R_F values are a maximum will be termed the "optimum speed"; the "optimum speed" in Table II is, therefore, about 450 r.p.m.

For several other solvents a similar set of runs was made at varying velocities and the same type of pattern was found as that shown in Table II. Instead of listing the whole gamut of R_F values obtained, Table III shows the R_F values at the "optimum speed". It is seen that this "optimum speed" is not constant for different solvents, but seems to lie nearer the lower velocities of rotation.

TABLE III

CHROMATOFUGE R_F VALUES OF ALGAL PIGMENTS AT "OPTIMUM SPEED"*

Conditions: Whatman No. 3 MM paper (unwashed); flow rate of 2.5 ml/min; 7.5 p.s.i. nitrogen pressure; liner saturated with 20 ml of solvent (unnecessary for toluene); time of development 10 min; circular origin, 10 cm. R_F values reported are the average of three separate runs at the velocities quoted.

	Solvent: toluene, "optimum speed" 600 r.p.m.		*Solvent: toluene–petroleum ether (4:1), "optimum speed" 500 r.p.m.*	
Compound	*Total extract*	*Carotenoid extract*	*Total extract*	*Carotenoid extract*
Carotenes	0.96	0.97	0.98	0.97
Lutein	0.89	0.90	0.90	0.90
Violaxanthin	0.82	0.80	0.78	0.74
Chlorophyll *a*	0.34	—	0.37	—
Chlorophyll *b*	0.19	—	0.27	—
Neoxanthin	0.04	0.06	0.07	0.07
	Solvent: toluene–petroleum ether (1:1), "optimum speed" 750 r.p.m.		*Solvent: toluene–petroleum ether (1:3), "optimum speed" 500 r.p.m.*	
Carotenes	0.98	0.94	0.98	0.95
Lutein	0.83	0.85	0.75	0.73
Violaxanthin	0.70	0.50	0.48	0.35
Chlorophyll *a*	0.33	—	0.30	—
Chlorophyll *b*	0.22	—	0.19	—
Neoxanthin	0.10	0.07	0.12	0.17

* "Optimum speed" is defined as that velocity of rotation of the chromatofuge where the R_F values of the pigments are a maximum.

Comparison of the R_F values listed in Tables II and III to those obtained in the conventional ascending or descending chromatography, shows the similarity obtained with the same solvents. In all cases carotenes run right at the solvent front. Solvents with a higher proportion of toluene are useful for the separation of the carotenoids; on the other hand, the chlorophyll *a* and *b* travel further in pertoleum ether–isopropanol solvents. Figs. 1 and 2 show the width of the pigment zones obtained; it is seen that the bands are very narrow and well-defined. They are less than one-half as wide as those which result from descending or ascending chromatograms, that have been run the same distance as the chromatofuge chromatograms.

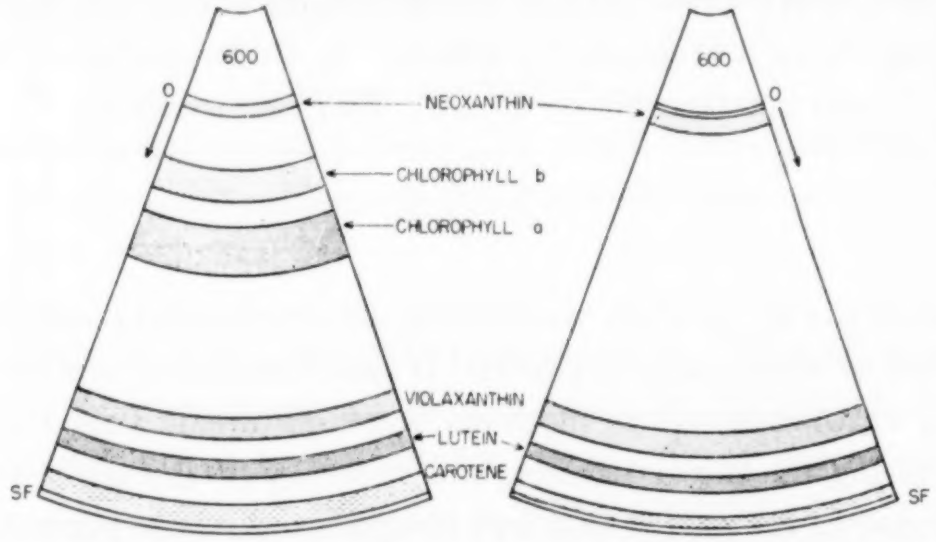

Fig. 1. Sectors of centrifugally accelerated paper chromatograms of the total pigment and carotenoid extracts from *Chlorella* using toluene as the solvent at 600 r.p.m. Time of development, 12.5 min; flow rate of 2.5 ml/min; origin → solvent front, 17 cm; Whatman No. 3 MM paper.

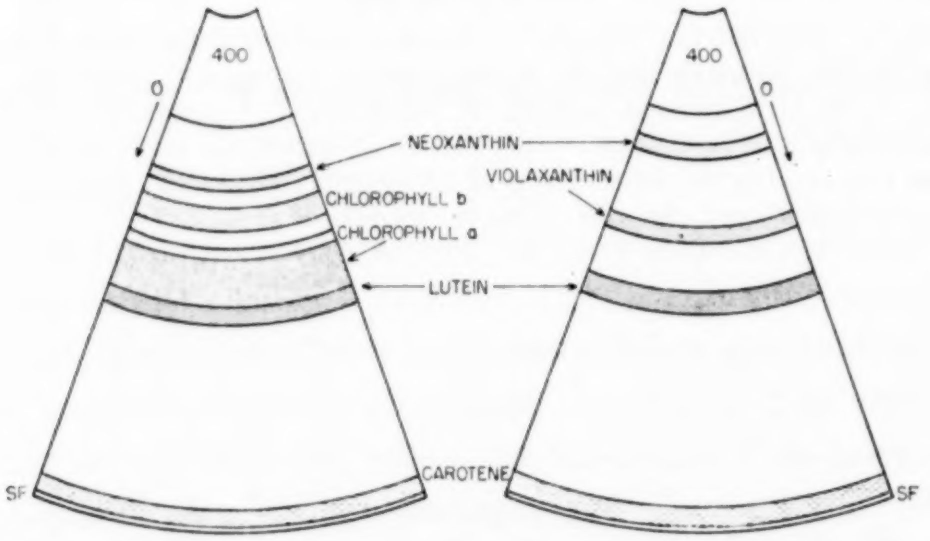

Fig. 2. Sectors of centrifugally accelerated paper chromatograms of the total pigment and carotenoid extracts from *Chlorella*, using petroleum ether–isopropanol (100:2.5) as the solvent at 400 r.p.m. Time of development, 10 min; flow rate of 2.5 ml/min; origin → solvent front, 17 cm; Whatman No. 3 MM paper.

In the hopes of obtaining better separation of chlorophyll *a* and *b*, reverse phase chromatography was tried. The methanol used as the solvent for the development of the paraffin oil-impregnated paper showed a great tendency to flood the papers. A flow rate of 1.25 ml/min with a 4 p.s.i. of nitrogen pressure was necessary, which meant at least 15 min development time. The results obtained were disappointing; the bands were diffused and streaking in to one another. All the pigments other than the carotene which stayed right at the origin had R_F values greater than 0.6, which meant that they were all crowded together despite the variety of conditions tried.

The quantitative recovery of pigments from a typical chromatogram was checked. About 88% of the pigments placed on the origin were recovered, which is much better than ascending chromatography where the average recovery was about 70 %[4]. Since the chromatograms take 10 to 15 min instead of 2 to 3 h to be developed, there is less time for decomposition of the pigments to occur. The pigment zones are easily eluted with ether and less time is needed to obtain colorless paper, hence there is a smaller loss in this operation than on the ascending or descending chromatograms. It would probably be advantageous to operate the chromatofuge at a lower temperature than 25°; it was not possible to do this with our machine.

SUMMARY

This paper presents the application of centrifugally accelerated paper chromatography for the separation of chloroplast pigments. It was found that similar results to conventional paper chromatographic methods were obtained. This technique appears to be very promising for the separation and recovery of the labile chlorophylls and carotenoids because of the narrowness and compactness of the pigment zones and the rapid development time. The pigments were easily eluted from the paper and a greater recovery of the pigments was obtained than from the usual paper chromatograms.

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BEITRAG ZUR DIREKTPHOTOMETRISCHEN AUSWERTUNG VON PHEROGRAMMEN AUF TROCKENFILMEN

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(Eingegangen den 18. November 1959)

Das unsaubere Arbeiten mit geölten Papieren und der Wunsch höhere Grade der Lichtdurchlässigkeit zu erreichen, als die bei normalen Papieren bestenfalls erreichbaren 20 %, haben Anlass gegeben, Methoden zu entwicklen, welche die Pherogramme als durchsichtigen Trockenfilm liefern. Ein Schritt in dieser Richtung war das von uns empfohlene Imprägnieren des Papiers mit Lacken von geeignetem Brechungsindex[1], wobei trockene Filme entstehen, deren Transparenzgrad dem geölter Papiere entspricht.

Es wurden weiterhin Methoden entwickelt, welche Pherogramme als durchsichtige Filme von sehr hoher Lichtdurchlässigkeit und gleichmässigem Untergrund liefern. Dieses Ziel lässt sich prinzipiell auf 3 Wegen erreichen:

A. Die Pherogramme werden von vornherein auf durchsichtigen Medien gemacht, welche nach Färben und Trocknen unmittelbar einen durchsichtigen Film geben; z.B. Agargel, Collodiumgel, Stärkegel.

B. Es wird auf Trägern elektropheriert, welche sich nachträglich in einen durchsichtigen Film umwandeln lassen. In Frage kommen hierfür besonders Träger aus Acetylcellulose, z.B. acetylierte Papiere, Membranfolien aus Acetylcellulose.

C. Normale, gefärbte Papierpherogramme werden mittels eines elektrophoretischen Kontaktkopierverfahrens auf einen durchsichtigen Film kopiert.

Es soll hier allein die Herstellung durchsichtiger Pherogramme behandelt werden. Die eigentliche elektrophoretische Technik wird nur insoweit berührt, als sie wichtig für die Herstellung solcher Pherogramme ist.

A. Durchsichtige Medien

Agargel. Die besonders von GRABAR und Mitarbeitern[2] ausgebaute Elektrophorese auf Agargel wurde von GIRI[3] technisch weiterentwickelt, indem er als Unterlage für das Agargel Cellophanmembranen oder Polyesterfilm verwendete; er bekam so trockene, durchsichtige Pherogrammfilme.

Als Träger hat sich bei uns eine mit einer Klebschicht aus Chlorkautschuk versehene PVC-Folie (Handelsname: Tesafilm No. 4) sehr gut bewährt. Wir spannen diese Folie unter Einrahmen mit Leukoplastband auf eine Glasscheibe auf und giessen die heisse, pufferhaltige Agarlösung auf die Klebschicht. Das erstarrte Agargel haftet gut auf dieser Chlorkautschukschicht und wird später beim Trocknen mit dem PVC-Film fest verklebt. Der Agarfilm wird zusammen mit der Glasplatte in die Elektrophoreseapparatur eingesetzt; die Stromzufuhr aus den Pufferträgen erfolgt in bekannter Weise über aufgelegte Brücken aus dickem Filterpapier. Bewährt haben sich Gele mit 0.5–0.75 % Agargehalt. Zusatz von 2–5 % 1,2-Propylenglykol erhöht die Geschmeidigkeit der Gele, unterdrückt die Bildung von Rissen und auch das Auskristallisieren von Puffersalz beim Trocknen; ferner wird dem Austrocknen des Gels während der Elektrophorese entgegengewirkt.

Steigende Agar-, Puffer- und Propylenglykolkonzentrationen wirken sich in einer Verlangsamung der elektrophoretischen Wanderungsgeschwindigkeiten bei erhöhter Zonenschärfe aus.

Zur Auftragung der Substanz legen wir eine aus einem ca. 0.5 mm dicken Glasfaden haarnadelförmig gebogene Glasspange auf das oberflächlich leicht angetrocknete Gel und lassen die Substanzlösung in die Spange einfliessen, wo sie kapillar festgehalten wird. Nach einigen Minuten wird sie mit einer Pinzette vom Gel abgehoben; hakenförmiges Abbiegen eines Schenkelendes erleichert dies. Die Lösung bleibt als scharfer Strich auf dem Gel liegen und diffundiert rasch vollständig ein.

Die gelaufenen Pherogramme werden zunächst auf der Glasplatte ausgespannt belassen und im Warmluftstrom bei ca. 35° getrocknet, wenn nötig anschliessend bei 80–90° hitzedenaturiert und dann von der Glasplatte abgenommen. Färbung in üblicher Weise; am besten aus 0.1 %iger Farbstofflösung in 95 %igem Methanol (2 % Eisessig enthaltend). Proteinpherogramme wurden gefärbt mit Amidoschwarz 10B, Neucoccin, Rubin S, Wasserblau 6B, Indigotetrasulfonat. Der Untergrund wird rasch und vollständig entfärbt.

Sollte sich die Agarschicht an einigen Stellen von der Unterlage abheben und sich etwas Flüssigkeit in einer Blase ansammeln, dann sticht man sie im Entfärbungsbad mit einer Nadel an. Der Blaseninhalt läuft aus und das Gel legt sich glatt an die Unterlage an; der Einstich ist nicht mehr sichtbar. Zum Trocknen spannt man den Film mit der Agarschicht nach aussen auf eine gewölbte Fläche, z.B. eine grosse Glasflasche auf.

Die Lichtdurchlässigkeit dieser Agarpherogrammfilme liegt zwischen 75 und 80 %. Die Proteinzonen sind stets klar durchsichtig. Bei Veronalpuffer, pH 8.6 ist die kathodisch gerichtete Endosmose sehr gross.

Collodiumgel. Das Prinzip besteht darin, aus einer Collodiumlösung und einem geeigneten Füllmittel (1,2-Propylenglykol, 1,4-Butylenglykol) eine Giessmasse herzustellen, die beim Verdunsten des Äthers zu einem klaren Gel erstarrt. Dieses Gel wird durch Baden in Pufferlösung vom Füllmittel, sowie von Resten Alkohol und Äther befreit. Man erhält klare. sehr wasserhaltige und doch steife Gele die beim Eintrocknen völlig durchsichtige Collodiumhäute geben. Als Träger für das Gel eignen sich Folien aus Celluloid oder Acetylcellulose, auf denen das Gel und später die Collodiumhaut gut haften.

Als Giessmassen haben sich bewährt:

(1) 40 ml Collodiumlösung (4 %ig) + 40 ml Butanol + 160 ml 1,2-Propylenglykol. Ausgewässertes Gel = ca. 0.8 %ig an Trockensubstanz.

(2) 20 ml Collodiumlösung (4 %ig) + 10 ml Butanol + 200 ml 1,4-Butylenglykol. Ausgewässertes Gel = ca. 0.3–0.4 %ig an Trockensubstanz.

Die Trägerfolie wird auch hier mit Leukoplastband auf einer Glasplatte ausgespannt und die Collodiummischung 1.5–2 mm dick in den Leukoplastrahmen auf die Folie gegossen. Nach 1 ½–2 Stunden ist die Masse zum Gel erstarrt. Nach Auswässern in Pufferlösung ist das Gel für die Elektrophorese bereit. Sowohl das frisch gegossene als auch das pufferfeuchte Gel sind einige Zeit (ca. 8 Tage) unverändert haltbar.

Die Elektrophorese selbst geschieht in bekannter Weise, wie z.B. bei Agargel (s.o.). Flüchtige Puffer sind vorteilhaft; bei hohen Konzentrationen an nichtflüchtigen Puffern können beim Trocken Puffersalze auskristallisieren und sich nach dem Färben auf dem Film als Abdrücke markieren.

Die getrockneten Pherogrammfilme werden von der Glasplatte abgezogen und wie üblich gefärbt. Der Untergrund nimmt überhaupt keinen Farbstoff an und lässt sich daher besonders leicht farblos waschen.

(a) Proteine werden in 0.1 %igen Farbstofflösungen in 0.1 *N* essigsaurem 50 %igem Methanol gefärbt (z.B. Neucoccin, Rubin S). Für Amidoschwarz 10B empfiehlt sich eine 0.01 %ige Farbstofflösung.

(b) Saure Mucopolysaccharide werden aus schwach essigsauren wässrigen Lösungen von Toluidinblau oder Brillantcresylblau angefärbt. Der Untergrund nimmt im Gegensatz zu Papier keinen Farbstoff an und wird leicht farblos gewaschen.

(c) Zur Sichtbarmachung von Aminosäuren badet man den Film 5–10 Minuten lang in einer Lösung von 300 mg Ninhydrin + 30 mg Cd-acetat in 30 ml *sec.*-Butanol und trocknet dann an der Luft. Nach kurzer Zeit setzt die Ausbildung der Rotfärbung der Aminosäuren ein.

Diese Pherogrammfilme sind völlig klar und durchsichtig. Die Lichtdurchlässigkeit liegt zwischen 84 und 88 % (400–600 mμ). Die Collodiumhaut ist trocken 5–15 μ dick.

B. Nachträglich in einen durchsichtigen Film umwandelbare Träger

Membranfolien (käuflich als "Membranfolien zur Elektrophorese"). Mit diesen aus Acetylcellulose hergestellten Folien erhält man besonders schöne Ergebnisse. Die elektrophoretische Technik entspricht vollständig der Papierelektrophorese, wobei horizontale Anordnung der Folien, z.B. in einer Apparatur nach LAURELL (sog. "Nagelbrett"), und ein Spannungsgefälle von 6–7 V/cm zu empfehlen sind. Die Pherogramme werden dann mit einem Gummirollenquetscher auf einer Glasplatte glatt aufgequetscht und bei Zimmertemperatur trocknen lassen. Auf eine Hitzedenaturierung kann bei vielen Proteinen verzichtet werden, sonst wird nur 5–10 Minuten bei 80° denaturiert. Längeres und höheres Erhitzen führt zum Kräuseln und Verziehen der Folien.

Die Färbung der von der Glasplatte herunter genommenen Folie geschieht wie bei Papierpherogrammen, wobei mit Leichtigkeit gute Anfärbungen bei völlig weissem Untergrund erhalten werden; z.B. Proteine mit den üblichen Eiweissfarbstoffen, Kohlehydrate mit der PAS-Färbung, Aminosäuren und Peptide mit Ninhydrin–Cd-acetat, saure Mucopolysaccharide mit Toluidinblau, ungesättigte Lipoide durch

Halogenaddition, Halogensilberbildung und anschliessende Reduktion zu Silber[4], Lipoide mit Sudanfarbstoffen.

Das gefärbte Folienpherogramm wird nach dem Auswaschen des Untergrundes nass auf eine Glasplatte mit dem Gummirollenquetscher aufgewalzt und mit Eisessig besprüht. Die Folie sintert zu einer durchsichtigen Schicht zusammen. Nach dem Verdunsten des Eisessigs ist der trockene Film bereits sehr lichtdurchlässig (80–83 %), zeigt aber noch eine geringe Trübung. Durch anschliessendes Besprühen mit Phenol–Wasser–Eisessig (80:20:10) wird er schlechthin absolut durchsichtig; im Warmluftstrom bei 35–40° verdunstet das Phenol in 1 ½–2 Stunden. Unter Wasser löst sich der Film leicht von der Glasunterlage. Er sieht aus wie die als Verpackungsmaterial dienenden "Cellophanhäute". 1–2 Stunden zwischen Filterpapier gepresst, bleiben die Filme völlig glatt.

Die Eisessig- und Phenolbehandlung schädigt die oben genannten Färbungen nicht. Der Einschluss der Farben in die Acetylcellulosemasse ist so vollständig, dass selbst von löslichen Farbstoffen unter Wasser nichts herausgelöst wird.

Da die Acetylcellulose bei der beschriebenen Durchsichtigmachung angelöst wird, lassen sich die Membranfolien dabei leicht verschweissen. Legt man beim Aufquetschen auf die Glassplatte 2 Folien mit den Schnittkanten aneinander, so verschmelzen diese beim Sprühen mit Eisessig usw. mit einander vollständig; die Verschmelzungslinie ist kaum sichtbar. Man kann daher Pherogrammfolien der Länge nach teilen, verschiedenen Färbungen unterwerfen (z.B. auf Proteine, Kohlehydrate etc.) und nachträglich wieder zusammen schweissen. Man bekommt so sehr eindrucksvolle Bilder.

Oft zeigen Proteinzonen, besonders bei höheren Konzentrationen, auf den durchsichtigen Pherogrammen neben der Färbung auch eine Trübung. Unter dem Mikroskop sieht man diskrete gefärbte Proteinpartikel in einer klaren farblosen Grundmasse. Die Trübung ist bei hohen Proteinkonzentrationen (z.B. Albuminzone der Serumpherogramme) auch im durchsichtig gemachten, ungefärbten Film sichtbar. Das Gleiche gilt auch für Acetylpapiere, die in Paraffinöl auf einen hohen Transparenzgrad (ca. 60 %) gebracht wurden; bei den an gewöhnlichen Papieren erreichbaren mässigen Transparenzgraden (ca. 14–17 %) fällt sie kaum auf. Ob und in welcher Stärke Trübungen auftreten, hängt von der Art des Proteins ab; bei klaren Proteinzonen zeigt das Mikroskop nur eine homogene Färbung des Films.

Trotz solcher Trübung bleibt bei der Direktphotometrie die lineare Beziehung von Dosis und Extinktionskurvenfläche erhalten. Die Eichkurven werden lediglich steiler.

Vollacetyliertes Papier. Die Technik des Durchsichtigmachens von Membranfolien lässt sich auch auf vollacetylierte Papiere anwenden. Man erhält auf diesen Papieren sehr gute Pherogramme. Besonders lassen sich darauf Glycoproteine bei völlig weissem Untergrund darstellen, wie HÖLZER[5] gezeigt hat. Bei Zusatz von 10 % 1,2-Propylenglykol zum Puffer benetzen sich die Acetylpapiere wie gewöhnliches Papier; es genügt auch schon dem Puffer etwas Aceton oder Alkohol zuzusetzen.

Beim Umwandeln in einen Trockenfilm muss vor allem der Einschluss von Luftblasen verhütet werden.

Das Pherogramm wird unter Evakuieren mit Anisol durchtränkt. Auf eine Glasplatte bringt man eine Flüssigkeitsschicht eines Gemisches von Phenol–Anisol–1,2-Propylenglykol (100:20:1.2) und legt das mit Anisol durchtränkte Pherogramm, *ohne es vorher abzulöschen*, auf diese Flüssigkeitsschicht. Dann giesst man weiterhin Phenol–Anisol–Propylenglykolgemisch darauf, bis das Papier vollständig damit bedeckt ist. Es wird sofort völlig durchsichtig. Dann wird wagerecht liegend im Warmluftstrom bei 35–40° getrocknet. Den Trockenfilm löst man unter Wasser von der Glasplatte ab.

Der Pherogrammfilm hat 68% Lichtdurchlässigkeit. Unter dem Mikroskop sieht man darin noch einige Faserreste, vermutlich einige nicht völlig acetylierte Papierfasern. Daher ist der Film auch nicht glasartig klar, sondern zeigt noch eine geringe Trübung. Aus dem 0.23 mm dicken Papier Schleicher u. Schüll 2043 a, vollacetyliert, entsteht ein 0.17 mm dicker Film.

C. Kopierverfahren

Elektrokopien von Papierpherogrammen. Legt man ein feuchtes, gefärbtes Papierpherogramm auf ein alkalisches Gel, so diffundiert rasch ein grosser Teil des vom Eiweiss gebundenen Farbstoffs in das Gel; die Farbstoff-Proteinbindung wird bei pH > 5–6 gelöst. Der zunächst unvollständige Farbstoffübergang wird durch Anlegen eines elektrischen Spannungsgefälles in Richtung Papier-Gel vervollständigt und es entsteht im Gel ein hinsichtlich Form und Farbstoffmenge getreues Abbild des Originals. Aus dieser Beobachtung wurde ein Kopierverfahren entwickelt.

Als Kopiermaterial dient eine mit Formalin leicht angehärtete Gelatine, die auch bei alkalischem pH nicht klebrig ist. Die daraus hergestellten Filme lassen sich trocken aufbewahren und quellen beim Einweichen in Pufferlösung wieder zu einem Gel mit den gewünschten Eigenschaften.

Man stellt unter Erhitzen auf dem Wasserbad eine wässrige Lösung her, die 20% einer harten, farblosen Gelatine und 3% Glycerin enthält. Wir benutzten die Gelatinesorte "Smaragd" der Fa. Heinr. Rathjen, Gelatinefabrik, Nienburg/Weser. Zu 100 ml dieser heissen Lösung gibt man unter gründlichem Durchmischen 10 ml einer 0.35%igen Formalinlösung (40%iges Handelsformalin 1:120 mit Wasser verdünnt).

Man giesst die heisse Gelatinemischung auf eine feuchte, auf einer Glasplatte unter Einrahmen mit Leukoplastband (sog. Lassoband) aufgespannte Cellophanfolie 2–3 mm dick auf. Für eine Fläche 30 × 50 cm werden ca. 500 ml gebraucht. Nach dem Erstarren wird im Warmluftstrom bei ca. 40° getrocknet und dann der Gelatinefilm samt der fest daran haftenden Cellophanfolie in passende Stücke zurechtgeschnitten.

Man quillt diese Gelatinefilme bei Bedarf in Pufferlösung (s.u.), wobei sich gleichzeitig die Cellophanhaut ablöst, die dann verworfen wird.

Die Apparatur für die Elektrokopie besteht aus

(1) einer Gleichstromquelle. Stufenlos regelbar bis maximal 20 V; belastbar bis 1–1.5 A.

(2) 2 Elektrodenblechen, entsprechend der Grösse der zu kopierenden Papierpherogramme, z.B. 20 × 6 cm. Anode = 0.05 mm starkes Pd-Blech. Kathode = 1 mm starkes Silberblech.

(3) 2 Kühlplatten aus Glas; mit Wasserkühlung.

Man wendet bei der Elektrokopie einen Ammonacetatpuffer vom pH 4.0 an, bei dem die Farbstoff-Eiweissbindung noch nicht gelöst und kein Farbstoff aus den gefärbten Zonen eluiert wird. Erst das an der Kathode entwickelte Ammoniak löst den Farbstoff vom Eiweiss ab, der dann sofort elektrophoretisch in das Gelatinegel abwandert. Der Ammonacetatpuffer wird durch Zugeben von konz. Ammoniak zu 0.2 *N* Essigsäure bis zum pH 4.0 hergestellt. Andere Puffersalze, z.B. Na-acetat, erwiesen sich als weniger gut geeignet.

Zur Ausführung der Elektrokopie legt man in der angegebenen Reihenfolge aufeinander:

Kühlplatte; Anodenblech aus Pd; 15–20 Lagen puffergetränktes Filterpapier (ca. 7 mm); Gelatinegel (ca. 1.2–2 mm); mit Puffer besprühtes Originalpherogramm; 2 Lagen pufferfeuchtes Filterpapier; Kathodenblech aus Silber; Kühlplatte.

An die Elektrodenbleche wird eine Spannung von 10–15 V gelegt. Anfangsstromstärke = 0.8–1 A, die im Laufe des Kopierens auf etwa die Hälfte absinkt. Mit Hilfe von zwischengelegten Streifchen aus dünner Pd-Folie wurden die Spannungsgefälle in den einzelnen Schichten gemessen (Tabelle I).

TABELLE I

Schicht	*Dicke mm*	*Spannungsgefälle V*	*V/cm*
kath. Deckblätter	0.75	2.2	29.4
Originalpherogramm	0.35	0.7	20
Gelatinegel	1.5	7.2	58
anod. Papierblock	7.1	5	7.05

Man kontrolliert den Fortgang des Kopierens, indem man nach 10 Minuten und dann in Abständen von 5 Minuten unterbricht, Kühlplatte und Kathodenblech abnimmt und das Originalpherogramm etwa zur Hälfte vom Gel abhebt, so dass es nachher wieder in die ursprüngliche Lage gebracht werden kann. Falls das Pherogram und das aufliegende Filterpapier zu trocken erscheinen, sprüht man mit Puffer nach.

Der Farbstoff wandert in etwa 12–15 Minuten quantitativ vom Papierpherogramm in das Gelatinegel. Nach etwa 45 Minuten hat er auch das Gel durchwandert und schlägt auf den anodenseitigen Papierblock durch.

Aus den für Papier und Gel gemessenen elektrophoretischen Wanderungsgeschwindigkeiten (Tabelle II) errechnen sich Kopierzeiten, die den experimentell gefundenen nahe kommen; nämlich 7.5 Minuten für den quantitativen Farbstoffübergang vom Pherogramm in das Gel und 55 Minuten bis zum Durchschlagen des Farbstoffs durch das Gel.

Diese Überschlagsrechnung berücksichtigt nicht die für die kathodische Ammo-

TABELLE II

WANDERUNGSGESCHWINDIGKEITEN (mm/V/h) VON NEUCOCCIN IN 0.2 *M* AMMONACETAT

pH	*Gelatinegel*	*Papier**
7.0	1.84	2.73
6.0	1.64	2.42
5.0	0.5	2.02
4.0	0.5	2.02

* Machery u. Nagel 214.

niakentwicklung und dessen Diffusion zum Pherogramm erforderliche Zeit. Ferner bleiben die im Gel herrschenden pH-Verhältnisse unberücksichtigt.

Die Kopie wird in folgender Lösung fixiert (ca. 20–30 Minuten): 84 Vol. Methanol (95 %ig) + 5 Vol. Glycerin oder 1,2-Propylenglykol + 10 Vol. Formalin (40 %ig) + 1 Vol. Eisessig.

Das Gel wird hierbei entwässert, die Gelatine stärker gehärtet, durch die saure Reaktion der Farbstoff auf der Gelatine fixiert. Der Glycerin- bzw. Propylenglykolzusatz dient als Weichmacher. Die nachgehärtete Gelatine schrumpft nicht beim Trocknen.

Man lässt den fixierten Gelfilm hängend an der Luft trocknen, bis er sich trocken anfühlt. Zum völligen Durchtrocknen quetscht man den noch sehr schmiegsamen Film auf eine Glasplatte, auf der er schwach anklebt. Nach dem Trocknen über Nacht wird er biegsam-elastisch und bleibt nach dem Abnehmen von der Glasplatte glatt. Die Elektrokopien sind bezüglich Form und Intensität der Zonen originalgetreu: Trübungen in den farbigen Zonen (s.o.) können hier prinzipiell nicht auftreten.

Alle elektrophoretisch wandernden Farbstoffe eignen sich für die Elektrokopie, besonders Farbstoffe mit Sulfosäuregruppen, z.B. Neucoccin, Brilliantcarmoisin, Indigotetrasulfonat. Eine Ausnahme macht Amidoschwarz 10B, weil es elektrophoretisch nur sehr langsam wandert (geprüft in 0.2 *M* Ammonacetat bei pH 8.0) und erst bei stark alkalischer Reaktion, pH > 10 vom Protein eluiert wird. Bei Bromphenolblau muss die Indikatoreigenschaft berücksichtigt werden.

Die elektrophoretischen Wanderungsgeschwindigkeiten sind etwas unterschiedlich, was sich in den Kopierzeiten ausdrückt. Indigotetrasulfonat > Neucoccin > Bromphenolblau.

Auch Anfärbungen mit sulfurierten Tripenylmethanfarbstoffen, z.B. Säurefuchsin (Rubin S) oder Tintenblau, lassen sich elektrokopieren. Die Farbstoffe wandern allerdings als ungefärbte "Farbbasen" in das Gel und werden erst beim Ansäuren der Kopien wieder sichtbar. Bei Zusatz von 2 ml 85 %iger H_3PO_4/100 ml Fixierbad treten die Farben rasch und haltbar wieder in Erscheinung. Die Unsicherheit in der Kontrolle des quantitativen Farbstoffübergangs spricht jedoch gegen die Anwendung dieses Farbstofftyps.

Beim Elektrokopieren wandert lediglich der Farbstoff in das Gelatinegel, das denaturierte Protein verbleibt auf dem Pherogramm und kann dort erneut angefärbt werden, so dass es wieder im ursprünglichen Zustand vorliegt. Es wird vorher etwa 30 Minuten gewässert, um eventuell anhaftende Gelatinepartikel abzuspülen.

Bei gut denaturierten Proteinen können vom gleichen Pherogramm mehrere gleichwertige Kopien angefertigt werden, die im Extinktionenschreiber gleiche Kurven geben. Zur Demonstration zeigen wir die Extinktionskurven von 2 aufeinander folgenden Elektrokopien von Serum- und Prolaktinpherogrammen (Fig. 1 und 2).

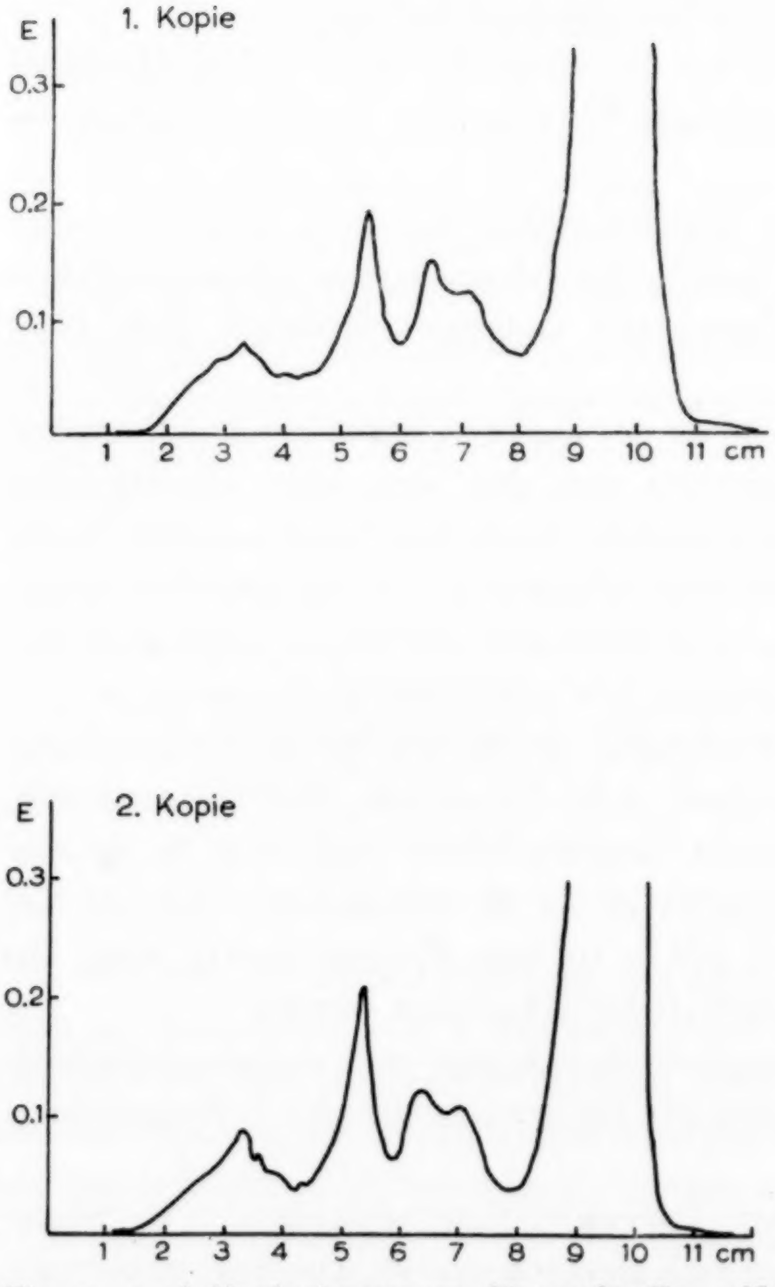

Fig. 1. 5 γl Kaninchenserum. Veronalpuffer, pH 8.6, 0.6 μ. Papier: Machery u. Nagel 214. Neucoccinfärbung. Extinktionskurven bei 522 mμ. Spaltlänge = Zonenlänge = 25 mm.

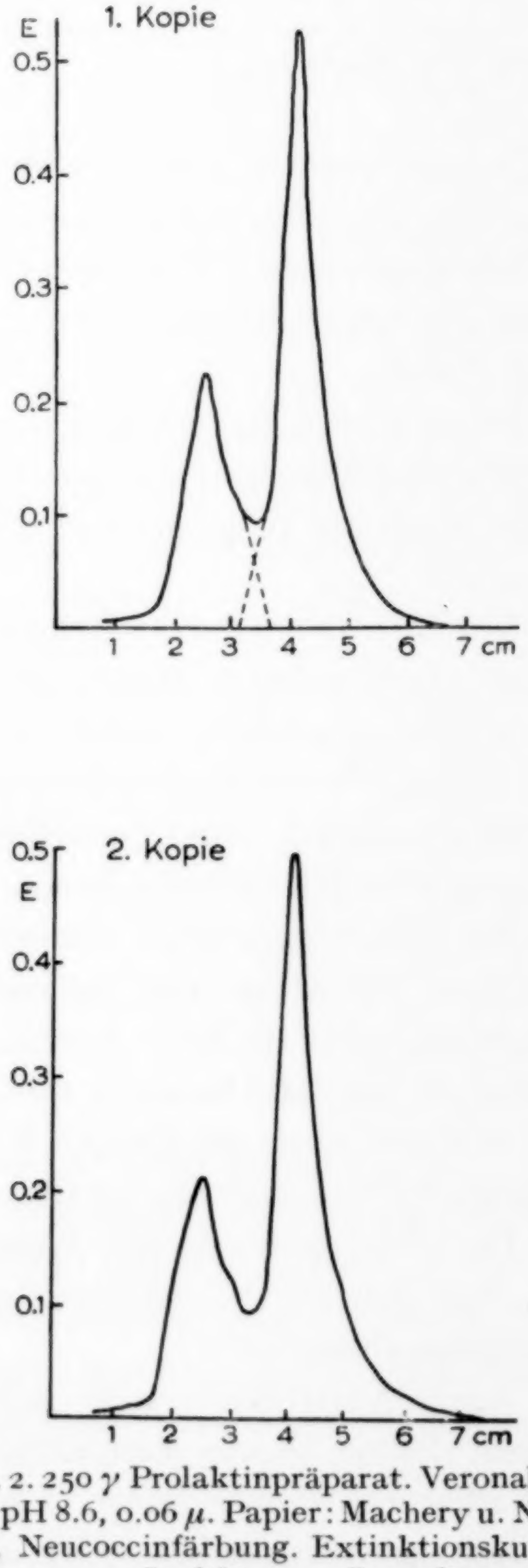

Fig. 2. 250 γ Prolaktinpräparat. Veronalpuffer, pH 8.6, 0.06 μ. Papier: Machery u. Nagel 214. Neucoccinfärbung. Extinktionskurven bei 522 mμ. Spaltlänge = Zonenlänge = 22 mm.

Die registrierten Kurven decken sich. Bei beiden Kopien des Prolaktinpherogramms ist das Verhältnis der Extinktionskurvenflächen 2.1:1. Das Prolaktin befindet sich in der schneller laufenden, stärkeren Zone.

ZUSAMMENFASSUNG

Für die Auswertung in Extinktionenschreibern werden Elektropherogramme als trockene, durchsichtige Filme hergestellt. Es werden 3 Wege zur Herstellung solcher Trockenfilme besprochen.

(1) Elektrophorese in durchsichtigen Gelen auf durchsichtigen Trägerfolien. Es wird ausser dem bekannten Agargel ein Collodiumgel verwendet, welches sehr durchsichtige Filme liefert.

(2) Pherogramme auf Trägern aus Acetylcellulose (Acetylpapier, Membranfolien) werden durch Lösungsmittel zu durchsichtigen Filmen gesintert. Die Durchsichtigmachung von Membranfolien wird durch Phenolspray verbessert, so dass völlig durchsichtige Filme entstehen.

(3) Es wird ein elektrophoretisches Kopierverfahren angegeben, bei dem der Farbstoff von einem Papierpherogramm auf einen durchsichtigen Film aus gehärteter Gelatine übertragen wird. Vom gleichen Original können mehrere Kopien angefertigt werden.

Es werden Arbeitsvorschriften für die Herstellung der durchsichtigen Pherogrammfilme gegeben.

SUMMARY

For purposes of evaluation in extinction recorders, electropherograms were prepared as dry, transparent films. Three methods for preparing such dry films are discussed.

(1) Electrophoresis in transparent gels on transparent supporting films. Besides the well-known agar gel, a collodion gel was used, which gave very transparent films.

(2) Pherograms on supports consisting of acetyl cellulose (acetylated paper, membrane films) were sintered to transparent films by means of solvents. The transparency of membrane films is improved by a phenol spray, completely transparent films being obtained.

(3) An electrophoretic printing method is described in which the stain is transferred from a paper pherogram to a transparent film of hardened gelatin. More than one copy can be made from the same original.

Procedures for preparing the transparent pherogram films are given.

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QUANTITATIVE INORGANIC CHROMATOGRAPHY

PART V. THE SEPARATION AND DETERMINATION OF FERROUS AND FERRIC IRON

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The chromatographic separation of the ions of a metal according to their valency states has received much less detailed attention than the separation of mixtures of different metal cations. Paper chromatographic separations have, however, been reported for antimony[1], arsenic[1-3], chromium[1,4], cobalt[1,5,6], copper[1,7-9], iron[1,7,10-13,25], manganese[9], mercury[1,7,9,12,14,15], molybdenum[1,12,16-18], plutonium[19], thallium[12,20], tungsten[21], uranium[11,22], and vanadium[1,6]. Ion exchange separation procedures have been described for iron[23], and uranium[24].

The chromatographic separation of complex mixtures of metals, prior to their estimation is now an established procedure, and in some instances provides serious competition to the more conventional analytical techniques. With the separation of valency states, however, quantitative methods of chromatographic analysis have been developed only for iron[13,25], molybdenum[16-18], and plutonium[19].

The aim of this part of the research was to develop a quantitative separation of the iron valency states by ion exchange methods based on the brief details mentioned by KRAUS AND MOORE[23]. To provide a suitable test for this separation, it was proposed to study an oxidation-reduction reaction (involving the iron valency states), whose reaction mechanism was already partially established and to compare the results. The latter aspect of the work is fully described in Part VI of this series.

EXPERIMENTAL INVESTIGATION INTO THE ANION EXCHANGE BEHAVIOUR OF FERROUS AND FERRIC IRON

For a particular resin, the quality of separation obtained depends upon each cation (or anion) having a sufficiently different affinity for, or adsorbability by, the resin, such that one cation is eluted while the other remains adsorbed on the column. The relative position of the exchange equilibrium for two exchangeable ions in a solution in equilibrium with an exchanger is a measure of the ability of the exchanger to differentiate the two ions, *i.e.* to adsorb one more than the other. This is usually measured by a separation factor K, which is conveniently defined as:

$$K = \frac{X_2}{X_1} \cdot \frac{C_1}{C_2} = \frac{D_2}{D_1} \tag{1}$$

where X_1 and X_2 are the mole fractions of the two species 1 and 2 in the resin phase, and C_1 and C_2 are the corresponding mole fractions outside the resin phase. D_1 and D_2 are the bed distribution coefficients of the ion species 1 and 2. (D = amount per ml of resin/amount per ml of solution.) Thus it can be seen that the greater this ratio K is from unity in any given system, then the easier the separation of the two ionic species. In practice, when $K > 1.3$, the species are separable by chromatographic procedures.

Unfortunately X and C are rather difficult to estimate by normal methods but D can be estimated in the following manner[26]. Define the elution constant E as

$$E = dA/V \tag{2}$$

where d = distance in cm which the maximum of an adsorption band travels after passage of V ml of solvent through a column of cross-section A cm². The velocity with which an adsorption band moves through the column under equilibrium conditions[27] is given as

$$d/t = v/(1 + D/i) \tag{3}$$

where t = time, v = linear flow rate of the solution, i = interstitial space, and D has its normal meaning.

Since

$$tv = V/iA \tag{4}$$

equation (3) reduces to

$$E = 1/(i + D) \tag{5}$$

When $D = 0$, *i.e.* species is unadsorbed

$$E \approx 1/i \tag{6}$$

When $D \gg i$, *i.e.* species is strongly adsorbed

$$E \approx 1/D \tag{7}$$

whence from equation (1) we may say

$$\frac{E_1}{E_2} = \frac{D_2}{D_1}$$

Hence if $E_1/E_2 > 1.3$ then a separation of the ionic species 1 and 2 should be possible.

When the solute is eluted from the column, obviously d is the length of the resin bed, and v the retention volume for that ion.

Thus, to develop a separation, the elution constants of both ferrous and ferric iron must be known over the whole range of solvents to be studied. Work was greatly simplified since KRAUS AND MOORE[23] state that the separation should be carried out in hydrochloric acid solutions using an anion-exchange resin. Since no data were available concerning the elution constants of the metal species under investigation on Amberlite IRA-400 in hydrochloric acid solutions, they were determined at 25° using a column technique as described below.

Experimental

Column

The column consisted of a double-walled Pyrex glass tube, the inner tube being 15 cm long and internal diameter 0.8 cm, fitted with a 2 mm glass tap at the lower end and a B.10 socket at the upper end. The water jacket was similar to a condenser jacket and was maintained at 25° by water being circulated through the jacket from a thermostat. The inner tube was fitted at the lower end with a No. 0 glass frit which supported 10 cm bed of − 150 to + 160 mesh Amberlite IRA-400 resin in the chloride form.

Procedure

The resin was purified by passage of about 200 ml of 1 *M* nitric acid, followed by copious washings with distilled water using a 50 cm head of liquid.

The resin was then saturated with hydrochloric acid solution by the passage of 250 ml of acid through the column after purification.

During elution of the column, the tap was opened and acid allowed to leave the column until the level fell to the top of the resin. The tap was closed and a small volume of the metal-bearing solution (0.2 ml delivered from an Agla micrometer syringe, containing 10.0 mg of metal per ml) in the same concentration of acid as that under investigation was placed on the column. The ferrous chloride solutions were prepared by dissolving pure iron powder in hydrochloric acid whilst the ferric chloride solutions were prepared from the AnalaR metal chloride. The tap was reopened and when the solution was completely adsorbed, fresh eluting agent was added and the elution commenced. The eluate was collected in 4 ml aliquots by means of an automatic fraction collector. Each fraction was evaporated to dryness and the residues dissolved in a small volume of dilute hydrochloric acid. These solutions were analysed by qualitative methods, the relative concentration of the metal ion present in each fraction being estimated by employing a standardised spot-test technique.

Results

The results of the experiments are summarised in Fig. 1. For ferric iron, adsorption became so strong with acid concentrations above 4 *M* that even by passage of a litre of eluting agent through the column, no ferric iron was detected in the eluate. Above 5 *M* hydrochloric acid, the results for ferrous iron became increasingly difficult to interpret since the majority of the ferrous iron is rapidly converted to ferric iron by autoxidation[28]. Above a concentration of 8 *M* acid, the ferrous iron was almost completely converted into iron(III) during the time of elution, hence no quantitative results were possible under these conditions (compare KRAUS AND MOORE[23]).

Using the data presented in Fig. 1, in 4 *M* hydrochloric acid solutions, $E_{\mathbf{Fe(II)}} = 2.0$, and $E_{\mathbf{Fe(III)}} = 0.05$.

Hence

$$K = 40$$

Thus an easy separation of ferrous and ferric iron should be possible under such conditions.

In the following section the analytical procedure is described for the quantitative separation of ferrous and ferric iron, and may be summarised as follows:

(a) The iron(II) and iron(III) were adsorbed in the anion exchange resin, as a solution of the mixed chlorides. The separation was carried out by elution with 4 *M* hydrochloric acid, followed by 0.5 *M* hydrochloric acid. The former reagent removed the ferrous iron, the latter the ferric iron. The iron content of each fraction was determined spectro-photometrically using 2-nitroso-1-naphthol-4-sulphonic acid[25].

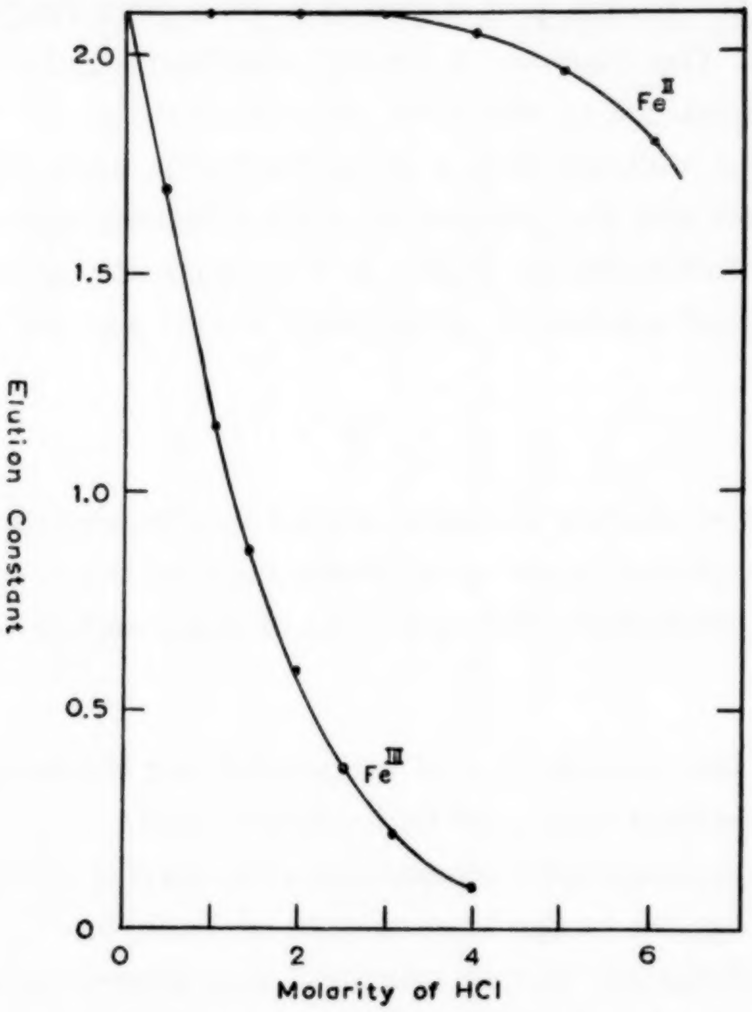

Fig. 1. Elution constants of ferrous and ferric iron in hydrochloric acid.

(b) Flow rate = 0.25 ml per cm^2 per min. Quantitative separation was obtained, however, the time taken to complete the elution was about 5 h, this was considered to be too long to be of use as an analytical method in kinetic studies. As a consequence, shorter columns were used in the investigation described below.

QUANTITATIVE SEPARATION OF FERROUS AND FERRIC IRON

1. *Preparation of the solutions for the columns*

Stock ferrous chloride solution (80 mg of iron/ml). 20.00 g of clean, dry iron were dissolved in the minimum of 4 *M* hydrochloric acid and diluted to 250 ml in a standard flask. The solution was stored under nitrogen in the normal manner.

The solution was standardised by titration with potassium dichromate solution in the presence of phosphoric and sulphuric acids, employing barium diphenylamine sulphonate as indicator.

1 ml of solution contains 79.8 mg of Fe(II).

A solution containing 25 mg of Fe(II)/ml was also prepared in a similar manner.

Stock ferric chloride solution (80 mg of iron/ml). 20.00 g of clean, dry iron wire was dissolved in the minimum of 4 *M* hydrochloric acid, and boiled with an excess of 100 volume hydrogen peroxide to oxidise the iron to the higher valency state. The solution was diluted to 250 ml, and stored in a Jena glass reagent bottle.

Mixed solutions. The (ferrous + ferric) standard solutions were prepared by pipetting suitable quantities of the stock solutions into 50 ml volumetric flasks and diluting to the correct volume with 4 *M* hydrochloric acid. The ratio of ferrous to ferric iron investigated and the volumes of stock solutions required for each standard mixed solution, are tabulated in Table I. The quantity of iron(II) and iron(III) present in 1 ml of mixed solution is given, since 1.0 ml was the major volume applied to the columns.

2. *Apparatus*

The apparatus used was exactly the same as that for the eluting constant experiments except that several columns were used. Each column consisted of a 10 cm bed of — 90 to + 100 mesh Amberlite IRA-400 resin of cross section area 1.65 cm^2.

3. *Procedure*

After purification of the resin with 1 *M* nitric acid and washing with distilled water, each column was saturated with 4 *M* hydrochloric acid.

At least two chromatographic separations were carried out for each of the volumes of "mixed solutions" given in the final column of Table I.

The required volume of "mixed solution" was placed on the top of the column and was given time to be absorbed; when this volume was less than 0.5 ml the solution was delivered from an Agla micrometer syringe. 25.0 ml of 4 *M* hydrochloric acid were allowed to percolate through the resin, the eluate being collected in a 25 ml volumetric flask. The eluting agent was changed to 0.5 *M* hydrochloric acid, and the following 25 ml of eluate were collected in a similar flask.

Aliquots of these fractions were taken and anlysed for iron content colorimetrically with 2-nitroso-1-naphthol-4-sulphonic acid[26]

The time taken for a complete analysis was approximately 1 hour, and it was considered short enough to allow the method to be applied for a study of slow reactions.

4. *Results*

Table II contains the results obtained using the method, while Table III contains the maximum errors obtained. The errors in Table III give an indication of the total error from the whole method, while Fig. 2 shows the type of separation obtained.

5. *Discussion*

Obviously when Fe(II) is in excess, some Fe(II) is converted into Fe(III), probably on passage through the column. Where Fe(II) is the major constituent, this interconversion is immediately noticed in the Fe(III) figure. Thus in 40 Fe(II):Fe(III) with 20 mg of Fe(II) present the error of 20 % in the Fe(III) result, corresponds to a conversion

TABLE I

Solution No.	*Ratio Fe(II)/Fe(III)*	*mg Fe(II) in*		*Vol. of 80 mg/ml soln. (ml)*	*Vol. of 25 mg/ml soln. (ml)*	*mg Fe(III) in*		*Vol. of 80 mg/ml soln. (ml)*	*Vol. of mixed solns. put on columns (ml)*
		1 ml	*50 ml*			*1 ml*	*50 ml*		
1	1:1	20	1000	12.5	—	20	1000	12.5	1.0
2		10	500	6.25	—	10	500	6.25	1.0, 0.5, 0.4, 0.2
3		1	50	0.625	—	10	50	0.625	1.0
4	1:4	5	250	—	10.0	20	1000	12.5	1.0, 0.2
5		2.5	125	—	5.0	10	500	6.25	1.0, 0.2
6	1:8	2.5	125	—	5.0	20	1000	12.5	1.0, 0.2, 0.1
7	1:40	1.0	150	—	2.0	40	2000	25.0	1.0, 0.25
8	1:100	0.5	25	—	1.0	50	2500	31.25	1.0, 0.5
9	4:1	20	1000	12.5	—	50	250	3.125	1.0, 0.5
10	40:1	20	1000	12.5	—	0.5	25	0.156	1.0

TABLE II

Ratio	*Iron present (mg)*		*Iron found (mg)*					
	Fe(II)	*Fe(III)*	*Fe(II)*	*Fe(III)*	*Fe(II)*	*Fe(III)*	*Fe(II)*	*Fe(III)*
1:1	20.0	20.0	19.88	19.90	19.95	19.90	—	—
	10.0	10.0	9.85	10.05	9.90	10.10	—	—
	5.0	5.0	5.05	5.009	5.10	4.90	—	—
	4.0	4.0	4.08	4.09	4.16	4.07	—	—
	2.0	2.0	2.00	2.05	2.04	2.02	—	—
	1.0	1.0	0.98	1.02	0.98	1.03	1.01	1.02
1:4	5.0	20.0	4.98	20.03	5.12	20.20	—	—
	2.5	10.0	2.46	10.10	2.50	10.30	—	—
	1.0	4.0	0.97	—	1.02	—	—	—
	0.5	2.0	0.49	2.02	0.49	2.02	0.51	2.13
1:8	2.5	20.0	2.51	—	2.48	—	—	—
	0.50	4.00	0.49	—	0.50	—	—	—
	0.25	2.00	0.24 (4)	—	0.25 (8)	—	—	—
1:40	1.0	40.0	0.99	—	1.00	—	—	—
	0.25	10.0	0.24 (8)	—	0.25 (1)	—	—	—
1:100	0.50	50.0	0.48	—	—	—	—	—
	0.25	25.0	0.25	—	—	—	—	—
4:1	20.0	5.0	—	4.97	—	5.12	—	5.15
	10.0	2.5	—	2.61	—	2.50	—	2.52
40:1	20.0	0.50	—	0.61	—	0.60	—	—

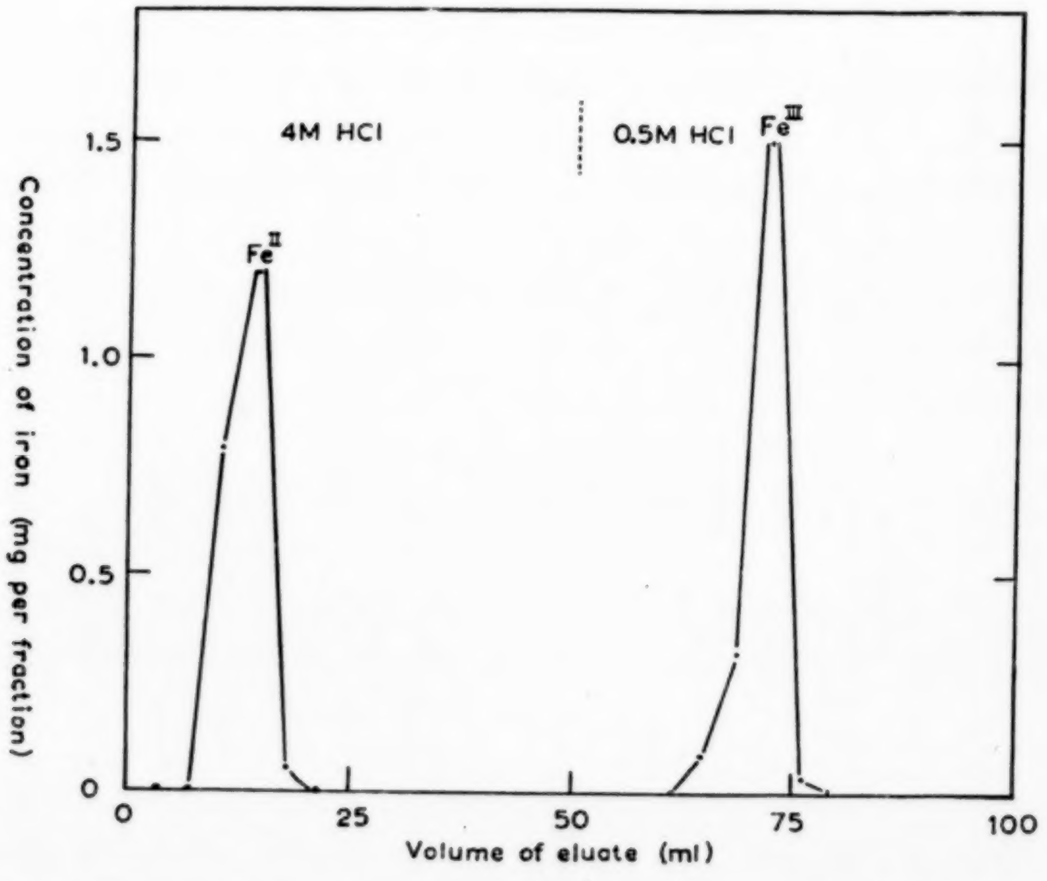

Fig. 2. The separation of ferrous and ferric iron on Amberlite IRA-400.

(neglecting all other errors) of only 0.5 % of the total Fe(III). POSNER[28] reported that in hydrochloric acid concentrations above 5 *M*, Fe(II) was rapidly autoxidised to Fe(III), while below 4 *M* acid the autoxidation was negligible. The rate of autoxidation is dependent upon the hydrochloric acid concentration and increases a hundred fold on increase from 4 to 7 *M* acid concentrations.

TABLE III

MAXIMUM ERRORS

Ratio 1:1	
1 %	5.0–20 mg of Fe(II) and Fe(III)
2.5 %	1.0–4.0 mg of Fe(II) and Fe(III)
Fe(III) in excess	
2 %	0.5–5.0 mg Fe(II) (1.4; 1.8)
2.5 %	0.25–1 mg Fe(II) (1.8; 1.40)
4 %	0.5 mg Fe(II) (1.100)
Fe(II) in excess	
2.5 %	2.5–5 mg Fe(III) (4:1)
20 %	0.5 mg Fe(III) (40:1)

By elution with 3 *M* hydrochloric acid for solutions with a high Fe(II):Fe(III) ratio, the error is greatly reduced:

Ratio Fe(II)/Fe(III)	*Fe(II) mg*	*Fe(III) mg*	*Fe(III) found mg*
40:1	200	0.50	0.52, 0.53

Unfortunately, however, the capacity of the column for ferric iron is reduced at this concentration of acid, and tends to be unadsorbed if the amount of Fe(III) exceeds 5 mg.

When Fe(II) is eluted with 5 *M* hydrochloric acid, then there is about a 3% conversion into Fe(III), the Fe(III) may be recovered by elution with 0.5 *M* hydrochloric acid.

SUMMARY

Using a simple anion-exchange technique, it was found possible to determine the minor constituent in mixtures of Fe(II) and Fe(III) iron in ratios between 1:100 and 40:1 respectively. The method showed maximum accuracy at ratios close to 1:1 (where both Fe(II) and Fe(III) were determined) and 5–20 mg of each valency state.

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CHROMATOGRAPHIC FRACTIONATION AND CHEMICAL CHARACTERIZATION OF SOME OLIGOSACCHARIDES SYNTHESIZED FROM LACTOSE BY *PENICILLIUM CHRYSOGENUM* THOM

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BALLIO, CHAIN AND DENTICE DI ACCADIA[1] reported the formation of reducing oligosaccharides from lactose by cell-free extracts of *Penicillium chrysogenum* and suggested that the main enzymic reaction was a transgalactosylation, a process which probably plays a primary role in determining the rate of lactose utilization by growing cultures of the mould.

It was felt necessary, in order to obtain a better understanding of this reaction, to determine the structure of the main oligosaccharides which the mould synthesizes from lactose. This paper describes a chromatographic method whereby the lower molecular weight sugars can be obtained, in quantities sufficient for structural studies, from culture filtrates of *P. chrysogenum* grown on lactose-containing media and reports the chemical characterization of the main oligosaccharides isolated by this technique.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were analytical grade products of C. Erba, Milan. Lactose used for the preparation of the corn-steep medium (92–93 % pure) was supplied by Polenghi-Lombardo, Rome, and corn-steep (50 % solids) by Fabbriche Riunite Amido Glucosio Destrina, Milan. Naphthoresorcinol was obtained from Mascia Brunelli Azzolini, Milan. Activated charcoal was a product of the British Drug Houses Ltd., England. Celite 503, supplied by Light & Co. Ltd., England, was washed with a large volume of 3 *N* hydrochloric acid, followed by distilled water until free of chloride ions, and was finally dried at 105°. Whatman's cellulose powder was the ashless standard grade for chromatography (W. R. Balston & Co. Ltd., England).

Organism

Penicillium chrysogenum Thom 49–133 Wis, or an isolate (51–20 c) of the strain 51–20 Wis were used.

Growth conditions

Both strains were grown in stirred fermenters[2], either on a corn-steep medium, or on the synthetic medium of JARVIS AND JOHNSON[3]. Media and growth conditions were the same as reported earlier[1,4], the only difference being a higher concentration of lactose (5 %) with the corn-steep medium.

Cell-free extracts

These were prepared from mycelium of the 51–20 c strain grown in stirred fermenters on the corn-steep–lactose medium. Their preparation, as well as the incubation procedure with lactose (48 h) have already been described[1].

Paper chromatography

Descending paper chromatography on Whatman No. 1 paper was used throughout. The solvents were: (a) pyridine–*n*-butanol–water, 4:6:3 by volume, with a 48 h developing time; (b) *n*-butanol–acetic acid–water, 4:1:5 by volume (top layer) with a 72 h developing time; (c) *n*-propanol–ethyl acetate–water, 4:2:4 by volume, with a 48 h developing time; (d) *n*-butanol–acetone–water, 2:7:1 by volume, with a 36 h developing time.

As a general spray for sugars a benzidine–trichloroacetic acid reagent was used[5]. The specific detection of reducing sugars was accomplished with a copper-phosphomolybdic acid reagent[6], and that of ketoses with a naphthoresorcinol–phosphoric acid reagent[7].

The rates of movement of the sugars are quoted relative to that of lactose (R_L) on the same chromatogram.

For purification purposes band chromatography on Whatman No. 3 MM paper was used.

Column chromatography

The column technique of WHISTLER AND DURSO[8] was used for the fractional separation of the sugars present in enzymic digests of lactose and in lactose-containing culture filtrates. Columns were packed with a slurry of activated charcoal mixed with an equal amount of acid-washed Celite 503 and the mixture of sugars, applied as an aqueous solution, was fractionated with ethanol by the gradient elution technique. The gradient was produced either by means of the closed system (constant-volume mixer) described in a previous paper[9], or with a simple apparatus by which the concentration of eluant varies linearly[10].

In one experiment the crude mixture of sugars was fractionated on a cellulose column. Before applying the sample, the column was washed with deaerated water, followed by the eluting solvent (isopropanol–*n*-butanol–water, 7:1:2, by volume).

Fractions were taken to dryness *in vacuo* in a rotatory evaporator[11] run at temperatures below 40°.

Hydrolyses

Chromatographically homogeneous oligosaccharides were hydrolysed with 1 *N* sulphuric acid for 2 h at 110° in sealed tubes. The hydrolysates were chromatographed on paper after neutralization with barium carbonate and centrifugation. Partial hydrolyses were carried out with 2 *N* acetic acid (110°, 7 h). Aliquots of these hydrolysates were spotted on paper without further treatment.

Analytical methods

The concentration of reducing sugars present in culture media or in partially purified mixtures was determined by SOMOGYI's method[12], using lactose as standard. Purified reducing oligosaccharides were assayed either by the volumetric procedure of WILLSTÄTTER AND SCHUDEL[13], as modified by WHISTLER AND TU[14], or by the colorimetric method of NELSON[15]. Fructose-containing oligosaccharides were assayed by the colorimetric method of ROE[16]. Monosaccharides obtained after acid hydrolysis were separated by paper chromatography, eluted with water and finally assayed by the colorimetric method of NELSON, according to the procedure outlined by BACON[17]. In all analytical determinations performed on compounds eluted from paper chromatograms, blanks prepared by elution of appropriate "clean" areas of the same sheet of paper were included. Optical rotations were determined at room temperature in aqueous solutions; the figures given are equilibrium values.

RESULTS

Paper chromatographic comparison of oligosaccharides produced from lactose by growing cultures, with oligosaccharides synthesized from lactose by cell-free extracts of P. chrysogenum

Paper chromatograms of the filtrates of *P. chrysogenum* cultures grown on lactose-containing synthetic or corn-steep media showed that several sugars other than lactose appeared during the fermentation. Two of them were readily identified as

TABLE I

COMPARISON OF THE PAPER CHROMATOGRAPHIC PATTERNS (SOLVENT a, BENZIDINE–TRICHLOROACETIC ACID SPRAY) OF SUGARS PRESENT IN CULTURE FILTRATES OF *Penicillium chrysogenum* GROWN ON LACTOSE-CONTAINING MEDIA, WITH THAT OF SUGARS FORMED ON INCUBATION OF LACTOSE WITH A CELL-FREE EXTRACT OF THE MOULD

R_L of main components	Culture filtrate of corn-steep medium	Culture filtrate of synthetic medium	Enzymic digest of lactose
0.47	+	+	+
0.68	+	+	+
0.81	—	+	+
1.00 (lactose)	+	+	+
1.35	+	+	+
1.65 (galactose)	+	+	+
1.90 (glucose)	+	+	+
2.10	+	+	—

The symbol — indicates that the compound has not been detected.

glucose and galactose. Others migrated on the paper at rates characteristic of the oligosaccharides synthesized from lactose by cell-free extracts of the mould (Table I).

Isolation of oligosaccharides from an enzymic digest of lactose

A 30 % solution of lactose (w/v, 40 ml) containing chloroform (2.5 ml) was incubated at 25° with a dialysed cell-free extract of *P. chrysogenum*, strain 51–20 c (40 ml). After 24 h incubation the mixture was heated 5 min in a boiling water bath and then rapidly cooled and centrifuged. The clear supernatant was adsorbed on a charcoal–celite column (volume 1700 ml, diameter 8 cm) and the sugars were eluted by a linear gradient of ethanol concentration (mixer containing 5000 ml of water and reservoir containing 5000 ml of 30 % ethanol) at a rate of 60 ml/h; fractions of 10 ml were collected. Aliquots of each tube were spotted on paper and developed with the benzidine–trichloroacetic acid reagent. Fractions giving a positive reaction were examined by paper chromatography. Fig. 1 shows the pattern obtained by plotting R_L values of individual fractions against the order of their emergence from the column. When groups of heterogeneous fractions were run on a second column of charcoal–celite, the separation was not appreciably improved, even by the use of more extended, flatter gradients of ethanol concentration. Appropriate groups of fractions, after being bulked and evaporated, were therefore further fractionated by band paper chromatography with solvent system a. Elution with water of sugar-containing areas of the chromatograms yielded, besides glucose (No. 2), galactose (No. 3) and lactose (No. 4), three oligosaccharides (No. 5, 8 and 10) in sufficient amount for further characterization, and another two (No. 7 and 11) in minute quantity. The occurrence of at least two components with low R_L values (0.22 and 0.15 in solvent a) was noted, but no attempt was made to isolate them.

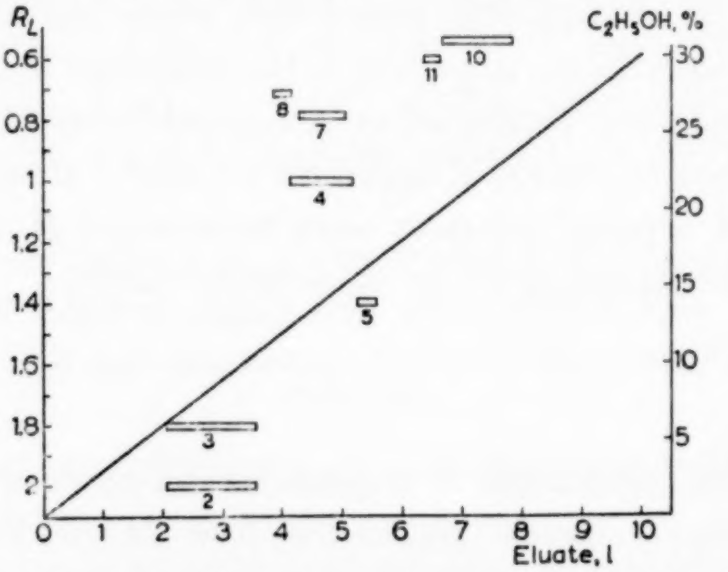

Fig. 1. Emergence from a charcoal–celite column of sugars formed by the action of a cell-free extract of *P. chrysogenum* on lactose. R_L is the ratio of the distance moved on paper chromatography (solvent a) by each substance, to that moved by lactose: the full line indicates ethanol gradient of effluent.

Isolation of oligosaccharides from culture filtrates

Synthetic medium. P. chrysogenum strain 49–133 Wis, was grown in a stirred fermenter, on the synthetic medium of JARVIS AND JOHNSON[3]. After 54 h, when the reducing sugars (expressed as lactose) assayed 5.8 g/l, 9 l were removed from the fermentation and filtered. The clear filtrate was concentrated *in vacuo* (below 40°) down to 1.2 l and divided into three 400 ml portions. Each portion was adsorbed on a charcoal–celite column (volume 1700 ml, diameter 8 cm) and the sugars were eluted, at a rate of 90 ml/h, by a linear gradient of ethanol concentration (mixer and reservoir as in preceding section). Fractions of 15 ml were collected and aliquots analysed according to the procedures described in the preceding section. As shown in Fig. 2, besides glucose (No. 2), galactose (No. 3) and lactose (No. 4), fructose (No. 1) and ten oligosaccharides were detected; purification by band paper chromatography yielded four of them (No. 6, 7, 8 and 10) in quantities sufficient for further characterization, and another two (No. 5 and 9) in minute amounts.

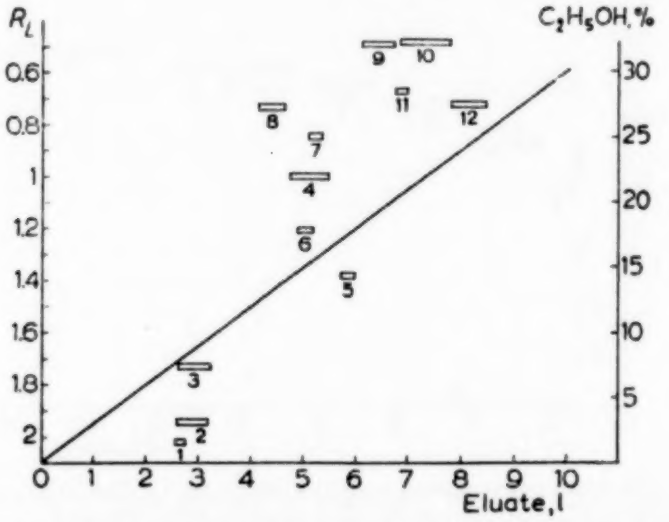

Fig. 2. Emergence from a charcoal–celite column of sugars present in the culture filtrate of *P. chrysogenum* grown on a synthetic lactose-containing medium. R_L is the ratio of the distance moved on paper chromatography (solvent a) by each substance, to that moved by lactose: the full line indicates ethanol gradient of effluent.

Corn-steep medium. P. chrysogenum, strain 51–20 c, was grown in a stirred fermenter on the corn-steep–lactose medium. After 40 h, when the reducing sugars (expressed as lactose) assayed 25 g/l, 10 l were removed from the fermentation and filtered. The clear filtrate was concentrated *in vacuo* to half its volume and the pH adjusted to 11.0 with saturated baryta. The heavy precipitate was removed by centrifugation, the pH of the supernatant was brought back to 7.0 with 5 *N* sulphuric acid and again centrifuged. The clear supernatant (6 l) was rapidly stirred at room temperature and 600 g of activated charcoal were added. After 3 h stirring, the charcoal was filtered, the clear solution analysed for reducing sugars and an aliquot chromatographed on paper. The filtrate contained 60% of the reducing sugars originally present (expressed as lactose). Paper chromatography indicated that these were mainly represented by lactose, glucose and galactose. The filtrate was discarded and the charcoal, after several washes with distilled water, was refluxed for 60 min with 3 l of 50% ethanol. After filtration the ethanol extraction was repeated and the combined filtrates were evaporated *in vacuo*, leaving a thick syrup which solidified when stirred with dry acetone. The solid was collected, weighed (44 g) and a portion (15 g) dissolved in water (20 ml). This solution was thoroughly mixed with cellulose

(45 g), dried *in vacuo* over calcium chloride, ground in a mortar to a fine powder and placed on the top of a cellulose column (volume 800 ml, diameter 5 cm). The sugars were eluted at the rate of 30 ml/h, fractions of 15 ml being collected. Aliquots of each fraction were analysed as described in the previous sections. Groups of homogeneous fractions were evaporated *in vacuo* and the oligosaccharides further purified, either by band paper chromatography, or by column chromatography on charcoal–celite columns. As shown in Fig. 3 chromatography on cellulose separated the mixture into 7 groups of fractions. The first and the second groups proved to be heterogeneous when analysed by paper chromatography (glucose plus galactose, and lactose plus two other components respectively). The occurrence of lactose and oligosaccharides No. 6 and 8 in the second group of fractions was indicated by chromatography on a column of charcoal–celite followed by paper chromatography. The sugars were isolated by band paper chromatography. As shown in Fig. 4 the third group of fractions could be resolved by chromatography on charcoal–celite into five oligosaccharides (No. 9, 10, 11, 12, 13). The fourth group of fractions was shown to be almost entirely composed of oligosaccharide No. 10. The groups of fractions that followed the fourth group

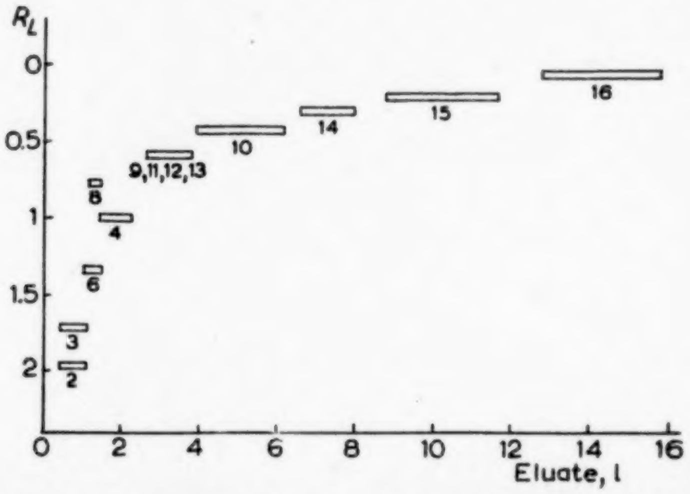

Fig. 3. Emergence from a cellulose column of sugars present in the culture filtrate of *P. chrysogenum* grown on the corn-steep–lactose medium. R_L is the ratio of the distance moved on paper chromatography (solvent a) by each substance, to that moved by lactose.

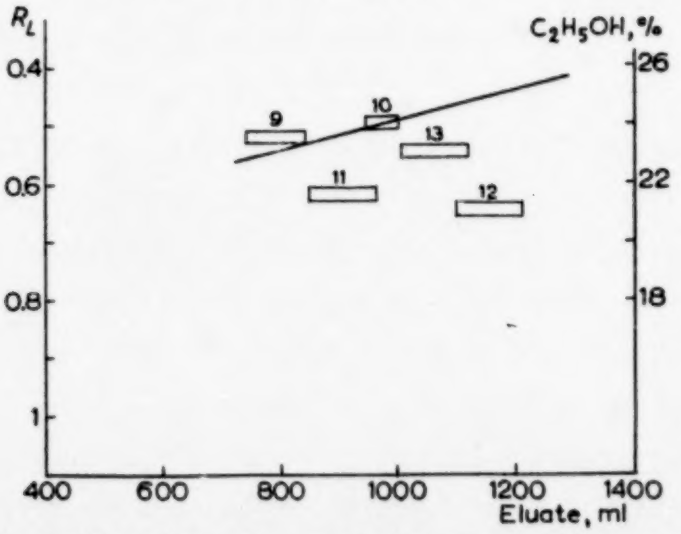

Fig. 4. Emergence from a charcoal–celite column of oligosaccharides present in a complex fraction, obtained by column chromatography on cellulose, of the culture filtrate of *P. chrysogenum* grown on the corn-steep–lactose medium. R_L is the ratio of the distance moved on paper chromatography (solvent a) by each substance, to that moved by lactose. The full line indicates ethanol gradient of effluent.

TABLE II

MOLECULAR WEIGHTS AND SPECIFIC ROTATIONS OF SOME OLIGOSACCHARIDES ISOLATED FROM CULTURE FILTRATES OF *Penicillium chrysogenum* GROWN ON LACTOSE-CONTAINING MEDIA OR FROM A LACTOSE SOLUTION INCUBATED WITH A CELL-FREE EXTRACT OF *Penicillium chrysogenum*

Compound No.	Probable structure	*Molecular weight*				*Specific rotation*				*Percent purity*					
		Calc.	*Found**			*Authentic compounds*	*1*	*2*	*3***	*On mol. wt. basis†*			*On [α]D basis††*		
			1	*2*	*3***					*1**	*2*	*3***	*1*	*2*	*3***
5	3-O-β-D-Galactopyranosyl-D-glucose	342	—	tr	396	+ 41.2°[19]	—	tr	+ 35.5°			86.5			86.5
6	x-O-β-D-Galactopyranosyl-D-fructose	342	420	410	—		— 33°	— 33.4°	—	81.5	83.5				
7	6-O-β-D-Galactopyranosyl-D-glucose	342	—	420	tr	+ 30.7°[23]	—	+ 27.3°	tr		81.5			89	
8	6-O-β-D-Galactopyranosyl-D-galactose	342	tr	420	400	+ 34.1°[24]	tr	+ 27.7°	+ 29.3°		81.5	85.5		81	86
9	O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→x)-D-fructose	504	600	tr	—		— 28°	tr	—	84					
10	O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→4)-D-glucose·$\cdot 2H_2O$***	540	560	600	586	+ 34°[18]	+ 33.8°	+ 32.7°	+ 32.4°	96.5	90	92	99	96	95.5
11	Unknown galactosyl-galactosyl-glucose	504	630	tr	tr		+ 32.7°	tr	tr	80					
13	Unknown galactosyl-galactosyl-glucose	504	556	—	—		+ 44°	—	—	90.5					

The symbol — indicates that the compound has not been detected among fractions from column chromatography, tr that it was available in too small amounts for analysis.

* Determined by hypoiodite oxidation, with the exception of those of compounds No. 6 and 9 which were calculated from the fructose content.

** Columns indicated with 1, 2, 3, report values for compounds isolated respectively from the corn-steep medium, the synthetic medium and the enzymic digest.

*** Analysis showed the compound to be a bihydrate[18].

† $\dfrac{\text{Calculated mol. wt.} \times 100}{\text{Found mol. wt.}}$

†† $\dfrac{[\alpha]_D \text{ of the isolated compound} \times 100}{[\alpha]_D \text{ of authentic compound}}$

TABLE III

PRODUCTS OBTAINED ON ACID HYDROLYSIS OF SOME OLIGOSACCHARIDES ISOLATED FROM CULTURE FILTRATES OF *Penicillium chrysogenum* GROWN ON LACTOSE-CONTAINING MEDIA, OR FROM A LACTOSE SOLUTION INCUBATED WITH A CELL-FREE EXTRACT OF *Penicillium chrysogenum*

Compound No.	*Isolated from the culture filtrate of corn-steep medium*		*Isolated from the culture filtrate of synthetic medium*		*Isolated from the enzymic digest*	
	Hydrolytic products	*Molar ratio*	*Hydrolytic products*	*Molar ratio*	*Hydrolytic products*	*Molar ratio*
5	—		Galactose and glucose	Not determined (tr)	Galactose and glucose	1.0:1.1
6	Galactose and fructose	1.0:0.9	Galactose and fructose	1.0:0.9	—	
7	—		Galactose and glucose	1.0:0.55	Galactose and glucose	Not determined (tr)
8	tr		Galactose		Galactose	
9	Galactose and fructose	1.0:0.4	Galactose and fructose	Not determined (tr)	—	
10	Galactose and glucose	1.0:0.55	Galactose and glucose	1.0:0.6	Galactose and glucose	1.0:0.55
11	Galactose and glucose	1.0:0.6	Galactose and glucose	Not determined (tr)	tr	
13	Galactose and glucose	1.0:0.6	—		—	

The symbol — indicates that the compound was not detected among the fractions from column chromatography, tr that it was available in too small amounts for analysis.

TABLE IV

PAPER CHROMATOGRAPHIC MOBILITIES (R_L) OF SOME OLIGOSACCHARIDES ISOLATED FROM CULTURE FILTRATES OF *Penicillium chrysogenum* GROWN ON LACTOSE-CONTAINING MEDIA AND FROM A LACTOSE SOLUTION INCUBATED WITH A CELL-FREE EXTRACT OF *Penicillium chrysogenum*

Compound No.	*Isolated from the culture filtrate of corn-steep medium*			*Isolated from the culture filtrate of synthetic medium*			*Isolated from the enzymic digest*			*Response to spray reagents*		
	Solv. a	*Solv. b*	*Solv. c*	*Solv. a*	*Solv. b*	*Solv. c*	*Solv. a*	*Solv. b*	*Solv. c*	*Benzidine-trichloroacetate*	*Copper-phosphomolybdic acid*	*Naphthoresorcinol*
5	—	—	—	1.30	1.32	1.45	1.32	1.35	1.43	+	+	—
6	1.23	1.49	1.42	1.23	1.50	1.40	—	—	—	+	+	+
7	—	—	—	0.81	0.87	0.86	0.83	0.88	0.86	+	+	—
8	0.76			0.75	0.86	0.78	0.75	0.85	0.78	+	+	—
9	0.58	0.63	0.45	0.56	0.63	0.44	—	—	—	+	+	+
10	0.52	0.52	0.42	0.51	0.52	0.40	0.52	0.52	0.41	+	+	—
11	0.64			0.66			0.65			+	+	—
12	0.71			0.72			—	—	—	+	+	—
13	0.61			—	—	—	—	—	—	+	+	—

The symbol — indicates that the compound has not been detected among fractions of column chromatography.

contained apparently homogeneous products (No. 14, 15, 16) which had low R_L values (0.32, 0.25 and 0.17 respectively in solvent a) and were not further examined.

These combined techniques resulted therefore in the detection of at least ten oligosaccharides. Five of them (No. 6, 9, 10, 11, 13) were obtained in sufficient amounts for further characterization.

Chemical characterization of individual oligosaccharides

Individual compounds shown to be homogeneous by both paper chromatography and charcoal–celite chromatography were further characterized by determining their molecular weight (by volumetric titration with alkaline hypoiodite or by the colorimetric methods of NELSON[15] and of ROE[16]), and their optical rotation (Table II); the nature and the molar ratios of the monosaccharides formed after acid hydrolysis were also established (Table III), and furthermore the paper chromatographic mobility in three solvent systems and the reaction to various spray reagents were determined (Table IV). In the case of some oligosaccharides isolated in very small amounts, some of these determinations were omitted. Crystallization of the compounds was never attempted.

On the basis of their chemical and physical properties and of their relative position in the elution pattern from charcoal–celite columns, it appears that most of the oligosaccharides synthezised from lactose by cell-free extracts are also formed by growing cells. These properties, when compared with those reported in the literature for β-galactosyl-oligosaccharides, provided sufficient evidence for a tentative identification of the main compounds isolated in the present study. Oligosaccharides No. 5, 7, 8 and 10 appear to be respectively 3-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-galactose and O-β-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→4)-D-glucose. The structure of oligosaccharide No. 8 is further supported by the results of direct paper chromatographic comparison in solvent systems a, b, c and d with a sample of authentic 6-O-β-D-galactopyranosyl-D-galactose kindly supplied by Dr. B. LINDBERG (Table V). The structure of oligosaccharide No. 10 is confirmed by the nature of the products formed by partial acid hydrolysis (Table VI) and by a recent study in which unambiguous chemical methods were used[18].

TABLE V

PAPER CHROMATOGRAPHIC MOBILITIES (R_L) OF OLIGOSACCHARIDE NO. 8 FORMED FROM LACTOSE BY *Penicillium chrysogenum* AND OF AUTHENTIC 6-O-β-D-GALACTOPYRANOSYL-D-GALACTOSE

Compound	*Solvent*			
	a	*b*	*c*	*d*
Oligosaccharide No. 8	0.68	0.72	0.72	0.62
6-O-β-D-Galactopyranosyl-D-gàlactose	0.68	0.72	0.73	0.62

In this table, R_L values of oligosaccharide No. 8 differ considerably from those reported in other tables, probably because a new batch of Whatman No. 1 paper was used.

TABLE VI

PAPER CHROMATOGRAPHIC MOBILITIES IN SOLVENT a, OF THE PRODUCTS OF MILD ACID HYDROLYSIS OF TWO TRISACCHARIDES FORMED FROM LACTOSE BY *Penicillium chrysogenum* COMPARED WITH THE MOBILITIES OF DISACCHARIDES FORMED FROM LACTOSE BY THE SAME ORGANISM AND OF SOME REFERENCE SUGARS

Compound	R_L	*Response to spray reagents*			*Probable structure*
		Benzidine-trichloroacetate	*Copper-phospho-molybdic acid*	*Naphtho-resorcinol*	
Trisaccharide No. 9	0.58	+	+	+	O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→x)-D-fructose
Hydrolysed trisaccharide No. 9	0.58	+	+	+	O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→x)-D-fructose
	0.73	+	+	—	6-O-β-D-Galactopyranosyl-D-galactose
	1.23	+	+	+	x-O-β-D-Galactopyranosyl-D-fructose
	1.65	+	+	—	Galactose
	2.10	+	+	+	Fructose
Trisaccharide No. 10	0.52	+	+	—	O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→4)-D-glucose
Hydrolysed trisaccharide No .10	0.52	+	+	—	O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→4)-D-glucose
	0.73	+	+	—	6-O-β-Galactopyranosyl-D-galactose
	1.00	+	+	—	Lactose
	1.65	+	+	—	Galactose
	1.90	+	+	—	Glucose
Disaccharide No. 6	1.22	+	+	+	x-O-β-D-Galactopyranosyl-D-fructose
Disaccharide No. 8	0.74	+	+	—	6-O-β-D-Galactopyranosyl-D-galactose
Lactose	1.00	+	+	—	
Galactose	1.65	+	+	—	
Glucose	1.89	+	+	—	
Fructose	2.11	+	+	+	

A partial structure can be suggested for oligosaccharides No. 6 and 9 which are both composed of galactose and fructose. As they give a positive reaction on paper chromatograms with the phosphomolybdic acid spray, but are not appreciably oxidized by hypoiodite, it follows that fructose must represent the reducing monosaccharide moiety.

The low specific rotation of disaccharide No. 6 and the well-known stereospecificity of enzymic transglycosylation reactions strongly suggest the β-configuration for the galactosidic linkage. At the moment the position of the linkage has not been established, but the behaviour on paper chromatography and the specific rotation are not much different from those reported for 3-O-β-D-galactopyranosyl-D-fructose[19]. It is fairly probable that trisaccharide No. 9 is the 6-O-β-galactopyranosyl-derivative of disaccharide No. 6, as indicated by the nature of the products formed by partial hydrolysis, *i.e.*, 6-O-β-D-galactopyranosyl-galactose and disaccharide No. 6 (Table VI). Ketoses having identical paper chromatographic mobility to those of the two abovementioned fructose-containing oligosaccharides were detected when a cell-free extract of *P. chrysogenum* was incubated with lactose in the presence of fructose (Table VII).

TABLE VII

PAPER CHROMATOGRAPHIC MOBILITIES OF TWO FRUCTOSE-CONTAINING OLIGOSACCHARIDES ISOLATED FROM CULTURE FILTRATES OF *Penicillium chrysogenum* GROWN ON LACTOSE MEDIA COMPARED WITH THOSE OF FRUCTOSE-CONTAINING OLIGOSACCHARIDES SYNTHESIZED BY A CELL-FREE EXTRACT OF *Penicillium chrysogenum* INCUBATED WITH A MIXTURE OF LACTOSE AND FRUCTOSE*

Compound	*R_L in solvent*		
	a	*b*	*c*
Oligosaccharide No. 6	1.30	1.53	1.34
Oligosaccharide No. 9	0.54	0.65	0.43
Reducing ketoses in the enzymic digest of lactose and fructose	0.55	0.65	0.44
	1.31	1.52	1.36
	1.95	2.90	2.62
Fructose	1.95	2.92	2.60

* A mixture of lactose (0.5 ml of a 30% aqueous solution) and fructose (0.5 ml of a 30% aqueous solution) was incubated 24 h at 25° with a cell-free extract (1 ml) of *P. chrysogenum* in the presence of chloroform (0.05 ml).

It thus appears that these ketoses represent true transgalactosylation products, which are produced in the lactose-containing media whenever fructose is also present. Fructose was indeed detected in culture filtrates of both the corn-steep and the synthetic media, whereas it was consistently absent in non-inoculated media; its appearance during growth of the mould is probably due to enzymic cleavage of some intracellular phosphate ester.

The specific rotations reported in this paper are generally lower than those quoted in the literature for authentic reference compounds. Inorganic contaminations from the charcoal–celite column might explain both this discrepancy and the high values found for the molecular weights. It should be noted that percentages of purity calculated independently, from specific rotations and from molecular weight values, are in good agreement with each other (Table II).

DISCUSSION

The results indicate that chromatography on columns of charcoal–celite followed by paper chromatography is a suitable process for the separation and purification of several oligosaccharides contained in the highly complex mixture of carbohydrates produced by *P. chrysogenum* from lactose. The use of gradient elution in the column chromatographic procedure gives a very reproducible and characteristic pattern and thus enables a preliminary identification of individual compounds to be made when it is applied to mixtures produced under different conditions. Evidence is yielded by this technique that most of the oligosaccharides present in culture filtrates of *P. chrysogenum* grown on synthetic and natural lactose-containing media are identical to those produced after incubation of lactose with cell-free extracts of the mould. Such an identity has been further demonstrated by comparing their paper chromatographic mobility, their optical rotation and the nature and molar ratios of the monosaccharides liberated by acid hydrolysis. As the preparation of large amounts of cell-free extracts of *P. chrysogenum* is tedious and time-consuming, the abovementioned findings are of importance when substantial quantities of galactosyl-oligosaccharides are required, *e.g.* for structural studies. In fact culture filtrates of *P. chrysogenum* grown on lactose are readily available, as they are obtained in large amounts in the industrial production of penicillin.

The structures assigned to the oligosaccharides so far examined yield further proof in support of the previous suggestion[1] that a transgalactosylation mechanism is operative when *P. chrysogenum* acts on lactose. A similar mechanism has been demonstrated with enzymes from other microorganisms[20]. In this respect it is of interest that three disaccharides produced from galactose by an enzyme of *Saccharomyces fragilis*[21], *i.e.* 3-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-glucose and 6-O-β-D-galactopyranosyl-D-galactose are also formed by *P. chrysogenum.*

The structural features of the oligosaccharides described in the present paper and the earlier findings[1] on the course of the enzymic reaction indicate that the transfer of the β-galactosidic moiety of lactose to primary alcoholic groups, *i.e.* the formation of β-1:6-galactosidic linkages, is predominant over that of other linkages. Similar observations have been made with other fungal transgalactosylases[22].

SUMMARY

Fractionation by column chromatography on charcoal–celite of β-galactosyl-oligosaccharides produced from lactose by *Penicillium chrysogenum* has shown that most of the sugars synthesized by cell-free extracts are also present in culture filtrates of the mould grown on lactose-containing media. These represent a suitable source for the isolation of oligosaccharides in amounts large enough for the study of their chemical structure. The characterization of 3-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-glucose, 6-O-β-D-galactopyranosyl-D-galactose, O-β-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→4)-D-glucose, as well as the probable structures of two oligosaccharides containing fructose and galactose are described.

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MONTAGE DE CATHAROMÈTRES À FIL DE TUNGSTÈNE POUR COLONNES DE CHROMATOGRAPHIE GAZEUSE

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INTRODUCTION

Afin de disposer de colonnes de chromatographie spécialement adaptées à nos besoins nous avons été amenés à en construire quelques unes nous-mêmes, ce qui nous a donné l'occasion d'acquérir une certaine expérience dans le montage des catharomètres à filaments utilisés comme éléments de détection. Nous décrivons ici les montages que nous avons adoptés.

Les considérations suivantes nous ont guidés dans le choix du métal du filament et des électrodes. Pour avoir une faible inertie thermique il faut prendre un filament aussi fin que possible. Il doit donc présenter une bonne résistance mécanique pour supporter les manipulations de montage. En outre parmi les métaux disponibles en fil très fin on a intérêt à choisir celui dont la résistance électrique a le plus fort coefficient de température. Enfin les électrodes doivent se souder parfaitement au pyrex, pour assurer une bonne étanchéité et une bonne rigidité mécanique. C'est ainsi que nous avons choisi le tungstène qui comme électrode se soude bien au pyrex et comme filament présente encore une solidité suffisante à 12 μ pour être manipulé à la main tout en restant visible à l'oeil nu. Nous avons éliminé le platine parce qu'il n'a qu'une résistance mécanique médiocre et ne se soude qu'imparfaitement au pyrex (les électrodes peuvent prendre un peu de jeu et occasionner un "bruit de fond"). En outre il peut présenter une activité catalytique indésirable dans certains cas (en particulier quand on l'utilise à température élevée). Dans l'emploi du tungstène la seule difficulté réside dans la jonction filament-électrode qui est difficile à réaliser directement. On verra plus loin comment nous l'avons surmontée. Le montage d'un catharomètre se fait en quatre étapes:

(1) Préparation du tube de verre.
(2) Préparation des électrodes.
(3) Préparation du filament.
(4) Montage proprement dit.

Nous décrivons le principe des différentes opérations dans leur suite logique. Notons dès à présent que toutes les dimensions concernant les catharomètres et les artifices de montage données ici ne le sont qu'à titre d'exemple.

CONSTRUCTION DE L'APPAREIL

(1) *Préparation du tube de verre*

La forme et les dimensions des tubes catharométriques sont pratiquement celles que ont été préconisées par DUPIRE ET BOTQUIN[1] (voir Fig. 1). Réalisés en principe par un souffleur de verre, ces tubes peuvent être faits avec un minimum d'adresse au laboratoire même. Il suffit de veiller à ce que leurs embouts capillaires soient bien coaxiaux (pour que le filament une fois monté soit bien dans l'axe du tube). Tout tube présentant un défaut à ce point de vue doit être éliminé car sa correction au cours du montage est malaisée et se termine généralement par la rupture du filament.

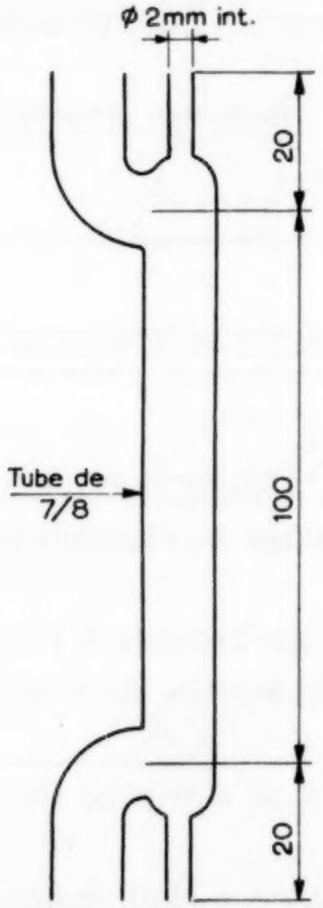

Fig. 1. Tube de catharomètre.

(2) *Préparation des électrodes*

Les électrodes sont en fil de tungstène de 1 mm de diamètre. On tronçonne du fil de tungstène en bouts de 40 mm de long. Dans cette opération on évitera autant que possible le fendillement longitudinal fréquent du tungstène. Puis on pratique à l'aide d'une meule un méplat sur une longueur de 2 à 4 mm à l'une des extrémités de l'électrode (voir Fig. 2). Le filament y sera soudé ultérieurement. On peut éventuellement se servir d'électrodes récupérées de catharomètres détériorés, mais il faut tenir compte de leur fragilité accrue par la soudure précédente.

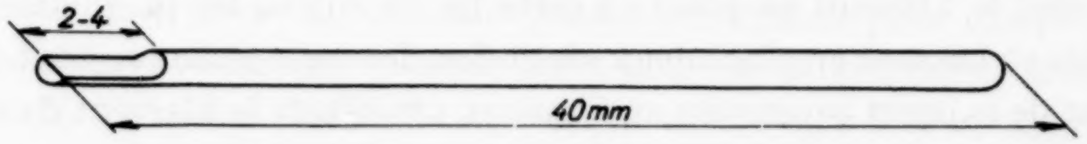

Fig. 2. Electrode de tungstène: vue du méplat destiné à recevoir le filament.

(3) *Préparation des filaments*

En raison de son extrême finesse le filament de tungstène (diamètre 12 μ) ne peut être soudé directement à l'électrode sans risque de détérioration. Nous avons trouvé que la meilleure façon de surmonter cette difficulté consistait à emprisonner ses extrémités dans la masse d'un métal suffisamment malléable se soudant bien au tungstène. Le nickel convient très bien à cet usage; l'opération nécessite le dispositif de la Fig. 3. Il se compose essentiellement d'une embase en dural présentant une rainure guide des électrodes (largeur 0.5 mm, profondeur 0.5 mm) et deux évidements (15 mm × 15 mm) dans lesquels on procède à l'emprisonnement du filament. Cette embase porte en outre quatre plaquettes de fixation des rubans de nickel et des électrodes.

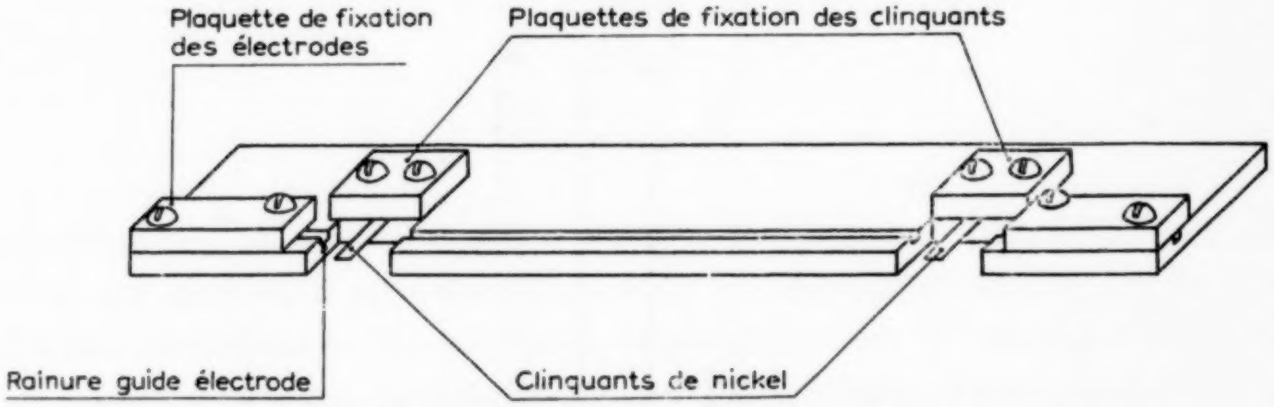
Plaquette de fixation des électrodes
Plaquettes de fixation des clinquants
Rainure guide électrode
Clinquants de nickel

Fig. 3. Dispositif de montage des filaments de catharomètre en verre.

La préparation des filaments se compose des opérations suivantes (voir Figs. 3 et 4).

(a) On découpe deux rubans de nickel, longueur plusieurs cm, largeur 2 mm, épaisseur 0.05 mm.

(b) Ces rubans sont mis en place à l'aide de leurs plaquettes de fixation respectives d'une manière telle:

que 10 mm environ dépassent dans les évidements;

que la distance entre les deux rubans soit toujours la même (100 mm dans notre cas). De cette manière on est assuré d'avoir des filaments de même résistance électrique (condition nécessaire pour se trouver au maximum de sensibilité du pont de Wheatstone dont ils feront partie ultérieurement).

(c) On met le filament en place: à cette fin on enlève les plaquettes de fixation des électrodes en laissant en place deux vis de fixation (une à chaque bout de l'embase) qui serviront de support provisoire au filament. On débite le filament de sa bobine en longueur suffisante et on le fixe momentanément à ces deux vis par quelques enroulements.

(d) On procède à l'emprisonnement du filament dans le ruban de nickel: pour cela on fait dans l'ordre les pliures successives montrées par la Fig. 4. À ce propos signalons qu'il peut être avantageux de faire la phase A de la pliure 1 avant la mise en place provisoire du filament sur les vis. Il est évident qu'il faut couper le filament avant la pliure 2.

(e) Après avoir fixé de cette manière le filament aux rubans de nickel, on met les électrodes en place. On s'assure que le méplat d'une électrode arrive exactement au-dessous du ruban de nickel, le filament se trouvant dans le prolongement de l'électrode.

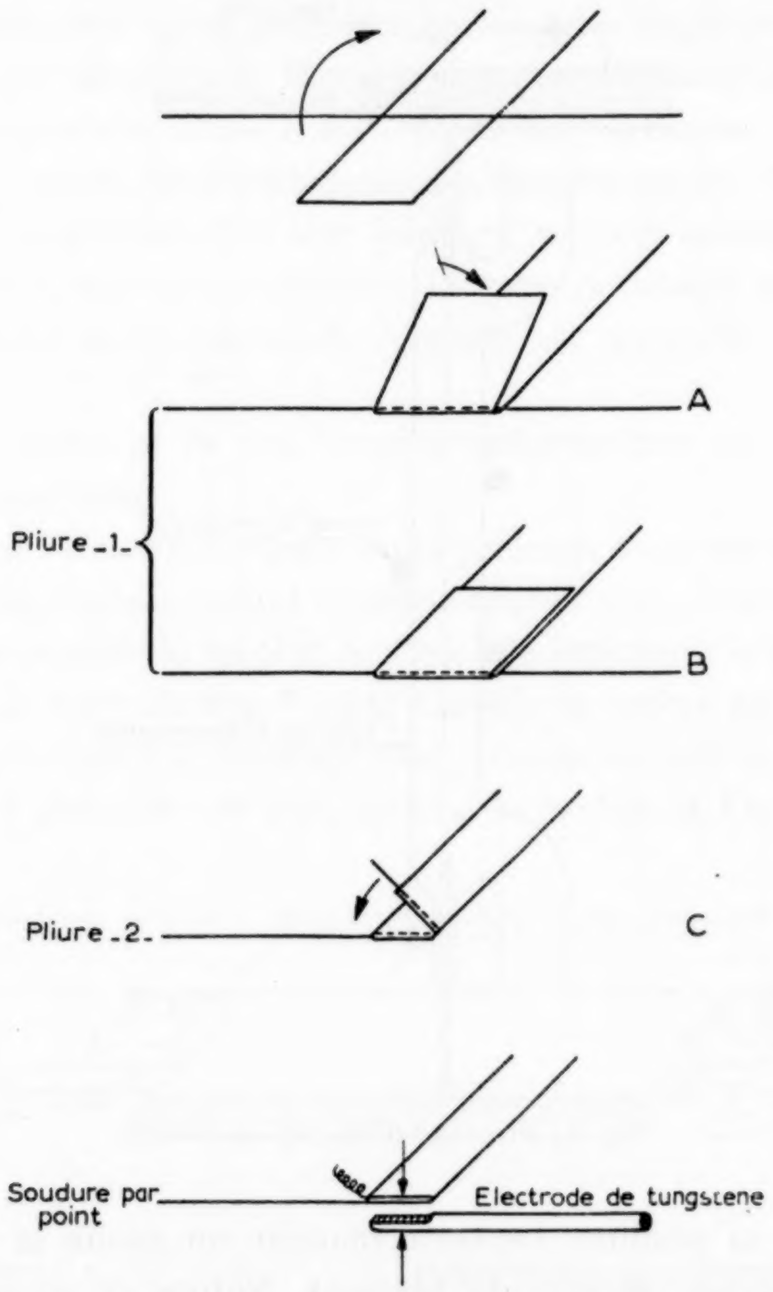

Fig. 4. Fabrication des catharomètres à filament de tungstène: suite des opérations de pliure et de soudure.

(f) Finalement on assemble le système filament/chape de nickel/électrode par soudure par point. Les conditions de cette soudure électrique (pression d'application, intensité et temps de passage du courant) sont à déterminer pour chaque machine à souder. La meilleure soudure est obtenue entre pôles de cuivre, car ce métal épouse très rapidement la forme des électrodes et évite de cette façon leur écrasement.

(g) Ensuite on libère le filament après avoir coupé le ruban de nickel au ras des électrodes à l'aide d'une lame de rasoir et on le conserve suspendu dans un tube à essai.

(4) *Montage des catharométres*

On procède au montage proprement dit du catharomètre à l'aide du dispositif schématisé par la Fig. 5. Le filament suspendu verticalement dans le tube correctement disposé est tendu par le poids de son électrode inférieure. À l'aide d'un chalumeau à main on soude sommairement au verre l'électrode supérieure A; puis on soude définitivement l'électrode inférieure B. On laisse refroidir et après avoir libéré l'électrode A, on retourne le tube catharométrique A se trouvant maintenant en bas du tube. Dans cette position on achève sa soudure. De cette manière on assure la régularité des deux soudures et une tension correcte du filament. Notons en passant qu'une soudure pyrex–tungstène est bonne quand le tungstène présente un aspect ambré à travers le verre.

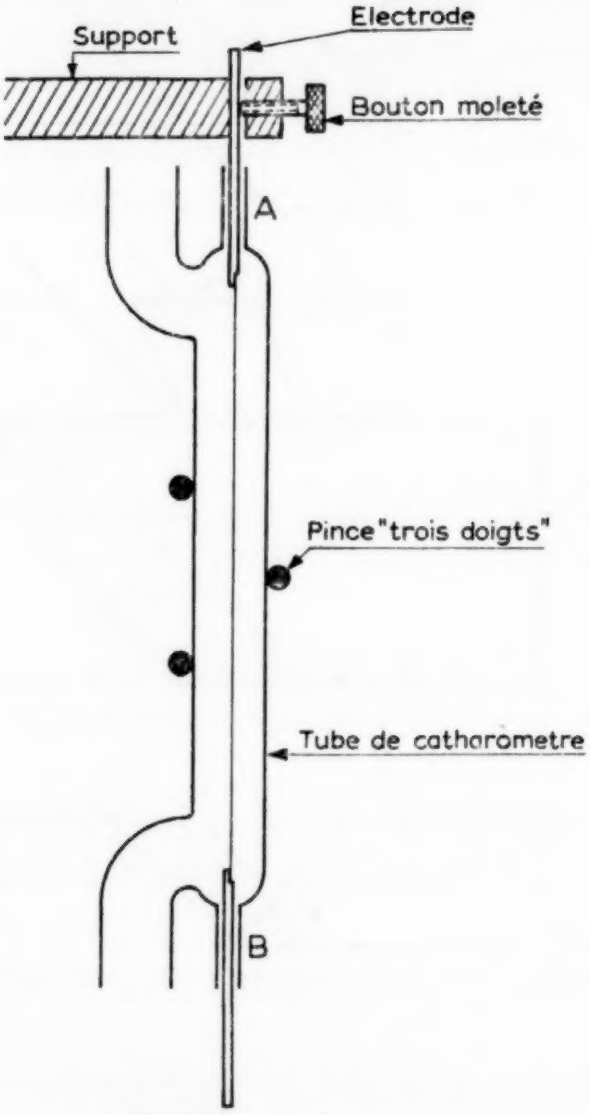

Fig. 5. Montage d'un catharomètre.

Pour rendre le catharomètre prêt à l'emploi on munit ses électrodes d'une couche de laiton à l'aide d'un fondant classique du type "CASTOLIN". De cette manière des jonctions électriques parfaites peuvent se faire directement soit par soudure à l'étain soit par brasure (cette dernière est à recommander chaque fois qu'on envisage l'emploi du catharomètre à des températures dépassant 200°). Finalement on mesure sa résistance pour permettre son classement au sein d'une série de catharomètres.

Les catharomètres montés ainsi ont généralement une résistance de 51 Ω et diffèrent rarement entre eux de plus de 0.4 Ω.

En opérant comme nous venons de l'indiquer on a rarement des échecs. Le point délicat reste toutefois l'introduction du filament dans le tube comme le montre la Fig. 5.

CAS DES CATHAROMÈTRES MÉTALLIQUES

Comme la méthode décrite ci-dessus a été appliquée avec succès à la réalisation de nombreux catharomètres en verre, nous avons pensé l'étendre aux catharomètres entièrement métalliques tels qu'on peut être amené à les employer dans les colonnes préparatives de grandes dimensions. Dans un appareil entièrement métallique il faut assurer une interchangeabilité facile des éléments, en particulier des catharomètres. A cette fin nous avons monté les filaments sur des bougies qui ont l'avantage d'assurer à la fois une bonne étanchéité (par leur joint) et un bon isolement des électrodes (mica) résistant à des températures élevées. Le type de bougie convenant le mieux à ce montage est celui dont l'électrode centrale est amovible donc modifiable à volonté.

Le principe du montage de ces bougies-catharomètres est un peu différent et comporte les phases suivantes:

(*a*) *Préparation de l'électrode centrale*: on la prolonge axialement en y brasant une courte tige de fer (1 cm environ) ayant le même diamètre que l'électrode à son extrémité—on y pratique ensuite le méplat destiné à la jonction électrode/filament.

(*b*) *Préparation du corps de bougie* (côté masse): on enlève au tour la ou les électrodes initiales qui gêneraient le montage final, ensuite on met en place une potence en fil d'acier (corde à piano de 0.8 mm) comme le montre la Fig. 6. La fixation est assurée par soudure.

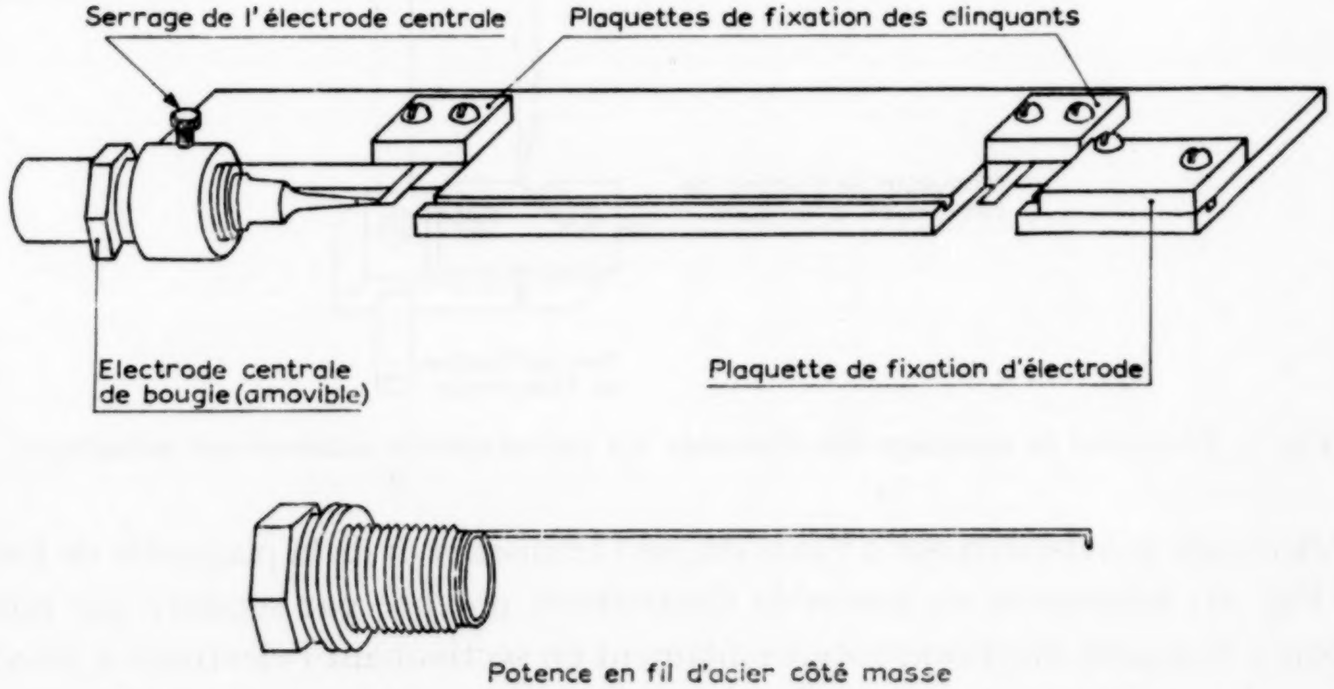
Serrage de l'électrode centrale
Plaquettes de fixation des clinquants
Electrode centrale de bougie (amovible)
Plaquette de fixation d'électrode
Potence en fil d'acier côté masse

Fig. 6. Dispositif de montage des filaments de catharomètre métallique.

(*c*) *Préparation du filament*: on procède comme pour un catharomètre en verre, la seule difference réside dans l'emploi de dispositif de la Fig. 6, analogue à celui de la Fig. 3, destiné à recevoir à une de ses extrémités l'électrode centrale de la bougie: on obtient ainsi l'électrode centrale avec son filament et l'électrode opposée (cette dernière n'a pas besoin d'être en tungstène, car il n'y a pas de soudure verre-métal).

(*d*) *Montage proprement dit du catharomètre*: il se fait à l'aide du dispositif de la Fig. 7. Ce dispositif étant fixé verticalement dans un étau, on commence par y visser le corps de bougie avec sa potence, puis on y engage avec précaution l'électrode centrale de haut en bas — on la serre définitivement dans le corps de bougie, en laissant pendre librement le filament et son électrode. La tension du filament ainsi assurée, on fixe l'électrode provisoirement à l'aide du pied coulissant et de la plaquette de fixation (voir Fig. 7); finalement on assemble électrode et potence par soudure par point et on libère l'ensemble électrode/potence/filament en sectionnant l'électrode à proximité de la soudure.

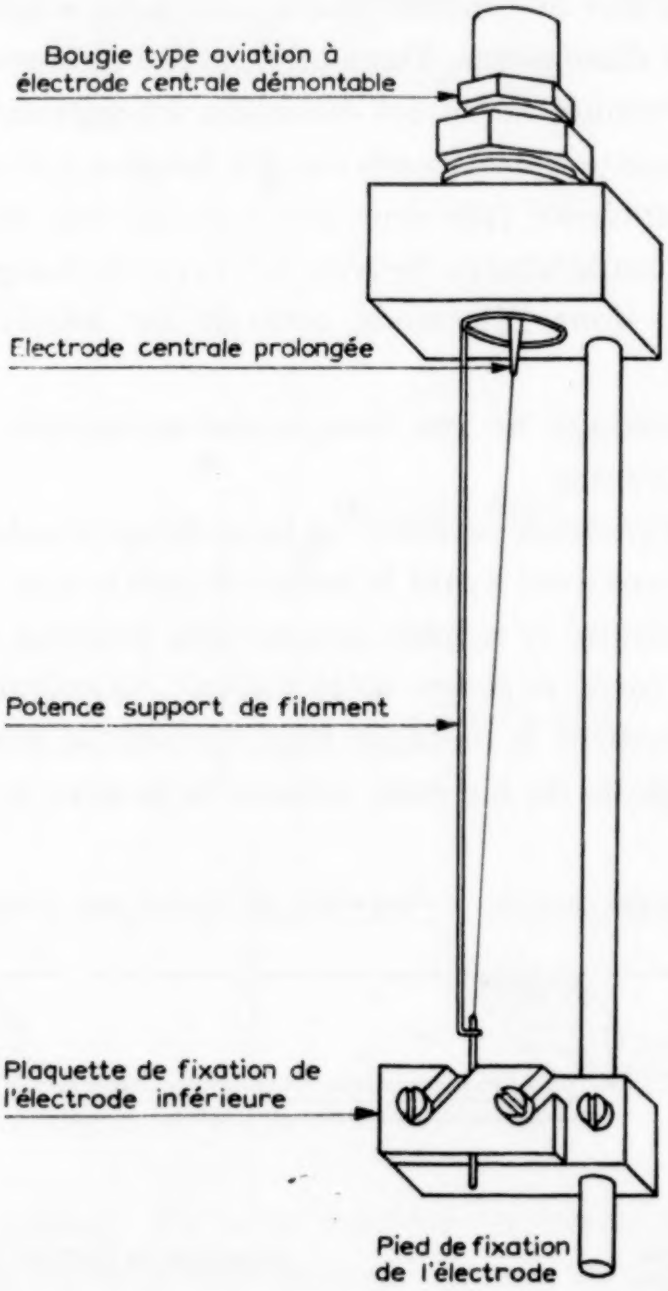
Bougie type aviation à électrode centrale démontable
Electrode centrale prolongée
Potence support de filament
Plaquette de fixation de l'électrode inférieure
Pied de fixation de l'électrode

Fig. 7. Dispositif de montage des filaments sur catharomètre entièrement métallique.

On dévisse les bougies de l'artifice de montage et on les met en place dans un bloc métallique prévu à cet effet comportant les alésages appropriés.

RÉSUMÉ

On décrit en détail le montage de catharomètres à filament de tungstène pour colonnes de chromatographie gazeuse. La première partie de la description est consacrée à la réalisation des catharomètres de colonnes en verre, de dimensions courantes. La

seconde partie concerne le montage analogue pour des colonnes entièrement métalliques de dimensions plus importantes.

SUMMARY

A detailed description is given of the construction of tungsten wire katharometers for gas chromatography columns. The first part of the description is devoted to katharometers for glass columns of ordinary dimensions. The second part deals with katharometers for columns made entirely of metal and of larger dimensions.

BIBLIOGRAPHIE

[1] F. Dupire et G. Botquin, *Anal. Chim. Acta*, 18 (1958) 282.

IDENTIFIZIERUNG ORGANISCHER VERBINDUNGEN

XXXVII. MITTEILUNG[*]

EIN BEITRAG ZUR PAPIERCHROMATOGRAPHISCHEN TRENNUNG DER ALKOHOLE ALS XANTHOGENATE

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Zur papierchromatographischen Trennung der aliphatischen Alkohole wurden von einigen Autoren[1–5] die Estersalze der Dithiokohlensäure, die Xanthogenate, als geeignete Derivate vorgeschlagen. Die Resultate der zitierten Autoren sind in der Tabelle I zusammengefasst. Aus dieser Tabelle ist ersichtlich, dass die Resultate einzelner Autoren übereinstimmen, in bestimmter Weise jedoch auffallend sind.

TABELLE I

R_F WERTE DER ALKYLXANTHOGENATE

Literatur	*1*	*2*	*3*	*4*		*5*
Lösungsmittelsystem†	*a*	*b*	*c*	*d*	*e*	*f*
Papier	—	—	*S & S 2043a*	*Whatman No. 1*		*S & S 2043b*
Methylalkohol	0.23	0.23	0.23	0.15	0.29	0.23
Äthylalkohol	0.35	0.35	0.35	0.25	0.45	0.33
n-Propylalkohol	—	—	0.55	0.38	0.65	0.44
Isopropylalkohol	0.44	0.44	—	0.38	0.61	—
n-Butylalkohol	0.55	0.55	0.71	0.52	0.77	0.55
Isobutylalkohol	0.54	0.55	—	—	0.77	0.54
n-Amylalkohol	—	—	0.79	0.58	0.79	—
Isoamylalkohol	0.62	0.62	—	0.56	0.79	0.57
Hexylalkohol	—	—	0.82	—	—	0.75
Octylalkohol	0.17	0.17	—	—	—	0.10
Nonylalkohol	—	—	—	—	—	0.14
Benzylalkohol	0.45	0.45	—	—	—	0.48
Cyclohexylalkohol	0.04	0.04	—	—	—	—

†a = *n*-Butylalkohol (2 % KOH).
b = *n*-Butylalkohol-5% $NaHCO_3$ (1:1).
c = *n*-Butylalkohol ges. mit Wasser, Papier imprägniert mit Phosphatpuffer pH 12.
d = *n*-Butylalkohol mit 2 % KOH.
e = wie d, Papier imprägniert mit KOH.
f = *n*-Butylalkohol ges. mit Ammoniak.

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[*] XXXVI. Mitteilung: *J. Chromatog.*, 4 (1960) 75.

J. Chromatog., 4 (1960) 138–143

In einer vorangehenden Mitteilung[6] aus unserem Laboratorium zeigten wir an praktischen Beispielen, welchen Regeln eine Trennung einer homologischen Reihe organischer Verbindungen folgen muss: im Falle einer polaren stationären Phase (in unserem Falle ist es Wasser) hat das Glied von kleinster Kohlenstoffatome-Anzahl den niedrigsten R_F Wert. Durch Umkehren der Phasen kann die Reihenfolge der *n*-Alkylhomologen umkehrt werden, auf keine Weise kann aber die Reihenfolge anders als $C_1 \rightarrow C_n$ oder $C_n \rightarrow C_1$ geändert werden. Weiter ist uns bekannt (siehe z.B. die 2,4-Dinitrophenylhydrazone der aliphatischen Carbonylverbindungen[7] oder die 3,5-Dinitrobenzoesäureester der Alkohole[8]), dass die cycloaliphatischen Verbindungen sich ähnlich wie die entsprechenden aliphatischen, um ein Kohlenstoffatom ärmeren Verbindungen verhalten. Deshalb hielten wir als unmöglich, dass in der homologen Reihe der Alkylxanthogenate, in welcher der R_F Wert vom Methyl- bis Hexylderivat von 0.23 bis 0.75 wächst, das Octylderivat den R_F Wert von 0.10 hätte. Auch die Angaben für das Cyclohexylderivat (Tabelle I) schienen uns unverständlich zu sein.

Wir reproduzierten deshalb die zitierten Methoden um diese Anomalien erklären und die Angaben bisheriger Autoren berichtigen zu können.

EXPERIMENTELLER TEIL

Darstellung der Kaliumxanthogenate

Einerseits stellten wir die reinen Xanthogenate mittels bekannter Methoden[9] dar und mittels Elementaranalyse prüften wir ihre Identität und Reinheit, anderseits überführten wir die Alkohole in die betreffenden Xanthogenate wie folgt:

Einige Tropfen des geprüften Alkohols wurden mit ca. 0.1 g gepulvertem Kaliumhydroxyd vermischt und erwärmt. Dann wurde 0.5 ml Schwefelkohlenstoff zugefügt, einige Minuten gründlich mit Glasstab durchgemischt und der Schwefelkohlenstoff durch Eintauchen des Reagensglases in warmes Wasser abgedampft. Der Rückstand wurde in einigen Tropfen Wasser unter gelindem Erwärmen (für Xanthogenate mit höheren Alkylen ist vorteilhafter Aceton zu benützen) gelöst und diese Lösung auf die Chromatogramme aufgetragen.

Lösungsmittelsysteme und Technik

Alle bisherigen Autoren benutzten zur Trennung der Xanthogenate alkalische Lösungsmittelsysteme, und zwar stets *n*-Butylalkohol in der Anwesenheit von Kaliumhydroxyd[1,4], Natriumbikarbonat[2], Ammoniak[5] oder Phosphatpuffer[3] von pH 12. Unsere Versuche führten wir mit folgenden Lösungsmittelsystemen durch:

n-Butylalkohol gesättigt mit:

(A) 2% KOH-Lösung

(B) 5% $NaHCO_3$-Lösung

(C) konz. Ammoniak

(D) konz. KCl-Lösung (Papier imprägniert mit 5% KCl-Lösung)

(E) konz. Na_2CO_3-Lösung (Papier imprägniert mit 5 % Sodalösung)
(F) Wasser
(G) 30 % K_2CO_3-Lösung (Papier imprägniert mit 5 % K_2CO_3-Lösung)
(H) wie G, Papier imprägniert mit 7.5 % K_2CO_3-Lösung
(I) wie G, Papier imprägniert mit 10 % K_2CO_3-Lösung.

Wir chromatographierten absteigend sowie aufsteigend auf Papieren Whatman No. 1, 2, 3 und WF 1 (Papierfabrik Niederschlag).

Sichtbarmachung der Xanthogenate

In den fünf zitierten Arbeiten werden insgesamt 18 Methoden zur Sichtbarmachung der Xanthogenate auf den Chromatogrammen vorgeschlagen. Unsere Versuche zeigten eindeutig, dass das Besprühen mit einer 10 %-igen Nickelsulfatlösung die einfachste, aber auch die geeignetste ist. Sie ist genügend empfindlich und die Chromatogramme (gelbe bis orangefarbene Flecken auf weissem Hintergrund) sind wochenlang haltbar.

Weiter benutzten wir zum Besprühen der Chromatogramme die ammoniakalische Silbernitratlösung (nach BERGNER UND SPERLICH[10]: 9 Teile 5 % $AgNO_3$-Lösung + 1 Teil konz. Ammoniak). Kurz nach dem Besprühen erschienen die Xanthogenate als gelbbraune, später schwarz werdende Flecken, die Zersetzungsprodukte der Xanthogenate als schwarze Streifen.

RESULTATE UND SCHLUSSFOLGERUNGEN

Aus unseren Versuchen, deren Resultate teilweise in Fig. 1 a–d zusammengefasst sind, können folgende Schlussfolgerungen gezogen werden.

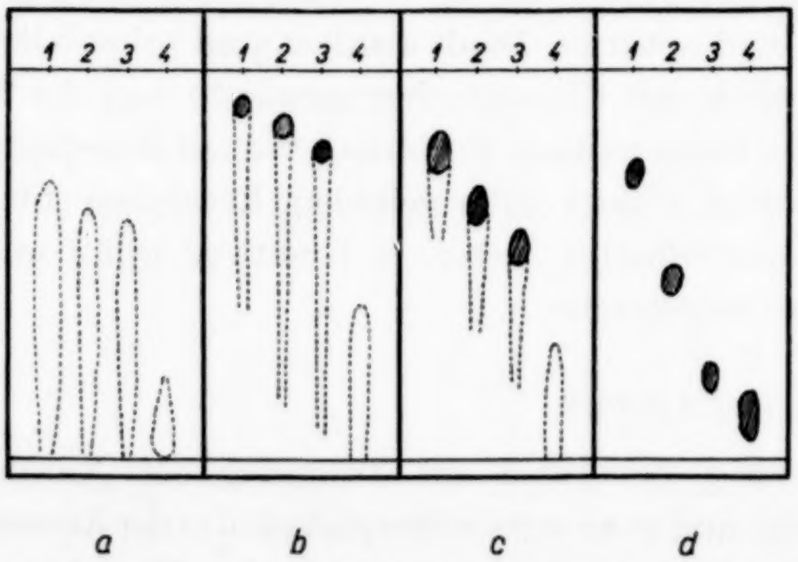

Fig. 1. Schema der Chromatogramme der Alkylxanthogenate. (1) Methylxanthogenat; (2) Äthylxanthogenat; (3) *n*-Propylxanthogenat; (4) Dodecylxanthogenat. (a) *n*-Butanol gesättigt mit Wasser; (b) *n*-Butanol gesättigt mit gesättigter KCl-Lösung, Papier imprägniert mit 5 % KCl-Lösung; (c) *n*-Butanol gesättigt mit konz. Ammoniak; (d) *n*-Butanol gesättigt mit 30 % K_2CO_3-Lösung, Papier imprägniert mit 10 % K_2CO_3-Lösung. ——— Alkylxanthogenate; — — — Zersetzungsprodukte der Xanthogenate.

In der homologen Reihe der Xanthogenate wächst der R_F Wert der Glieder mit C_1–C_{12} Alkylen, wie zu erwarten war, proportional mit der Länge des Alkylrestes.

Dies ist geltend auch für die C_8 und höhere Alkylderivate und auch das Cyclohexylderivat verhält sich ähnlich wie das Amylderivat, also ganz analog anderen Klassen homologischer Verbindungen. In diesem Sinne sind die Resultate von KARIYONE, HASHIMOTO UND KIMURA[1, 2] und PREY, KABIL UND BERBALK[5] (Tabelle I) zu korrigieren.

Während unserer Studie konnten wir jedoch solche Beobachtungen machen, die uns zur Frage führen, ob die Xanthogenate überhaupt geeignete Derivate zur Papierchromatographie sind. Bei der Anwendung der Lösungsmittelsysteme A–D und F zeigte sich nämlich, dass die Xanthogenate in neutralen oder nur schwach alkalischen Lösungsmittelsystemen verdoppelte und verwaschene Flecken bilden (System A, B), oder dass auf den Chromatogrammen manchmal überhaupt kein Xanthogenat sichtbar gemacht werden kann (System C, D, F). Deshalb benützten wir neben der Sichtbarmachung mit Nickelsulfatlösung noch die ammoniakalische Silbernitratlösung zur Detektion der Zersetzungsprodukte der Xanthogenate. Den bisherigen Angaben der Literatur[9] nach sollen die Alkalisalze der Xanthogensäure zwar stabil, dagegen die freien Xanthogensäuren unstabil sein. Wir fanden jedoch, dass bei der Papierchromatographie in ungenügend alkalischen Lösungsmittelsystemen in jedem Falle mehr oder weniger die Zersetzung eintritt; die Zersetzung der Xanthogenate ist im neutralen Lösungsmittelsysteme F vollkommen, sodass nur die Zersetzungsprodukte sichtbar gemacht werden können (siehe Fig. 1 a–d); mit zunehmender Alkalität des Lösungsmittelsystemes nimmt sie ab und in stark alkalischen Systemen G, H, I, E wandern die Xanthogenate praktisch ohne Zersetzung. Die Form der Flecken und die Tatsache, dass die Zersetzung von der am Chromatogramm zurückgelegten Laufstrecke abhängig ist, führt uns zur Meinung, dass hier durch Dissoziation und Hydrolyse die freien Xanthogensäuren entstehen, die sich weiter zersetzen. Einen Beweis dafür finden wir darin, dass die Zerzetsung der Xanthogenate schon durch Anwesenheit von KCl (neutrales Medium!) vermindert wird (Fig. 1b), was wir durch Unterdrückung der Hydrolyse erklären. Der Vorgang ist analog demjenigen, den wir im Falle aliphatischer Amine[11] eingehender studierten. Neulich erschien die Mitteilung von PLASKIN UND OKOLOVIČ[12], die unsere Beobachtungen zu unterstützen scheint. Diese Autoren studierten das Verhalten der Alkalixanthogenate in wässrigen Lösungen und fanden, dass diese desto weniger stabil sind, je verdünnter die Lösung ist.

Bei Anwendung der stark alkalischen Lösungsmittelsysteme G, H, I trat nicht nur keine Zersetzung ein, sondern wir erhielten eine gute Trennung der ersten drei Glieder der homologen Reihe. Diese Resultate stimmen überein mit denen von DYR, KRUMPHANZL UND ŠÍCHO[4], nur finden wir vorteilhafter das Papier mit Potaschelösung zu imprägnieren, da Kalilauge das Papier zerstört[4] und sowieso Kohlendioxyd abfängt. Es sei noch darauf hingewiesen, dass durch den Imprägnierungsgrad des Papieres die R_F Werte und die Trennung der Xanthogenate beeinflusst werden und zwar mit zunehmendem Gehalt des Papieres an Kaliumkarbonat werden die R_F Werte vergrössert.

Die Zersetzung der Alkalixanthogenate beobachteten wir auch während des

Aufbewahrens der wässrigen oder acetonischen Lösungen. Besonders war dies der Fall beim Cyclohexyl und höheren Alkylxanthogenaten.

Weitere Komplikationen können Spuren von Schwermetallen im Papier herbeiführen. Es werden Schwermetallsalze der Alkylxanthogenate gebildet, die langgezogene gelbe Streifen bis zur Lösungsmittelfront bilden, das Alkalixanthogenat "verbrauchen" und man kann dann keine Xanthogenate mehr sichtbar machen. Dieses Phänomen beobachteten wir bei Anwendung des Papieres WF 1 (Papierfabrik Niederschlag) und fanden sogleich, dass dieses Papier beträchtliche Spuren von Eisen und Kupfer enthält.

Alle diese Beobachtungen zeigen, dass die Anwendung der Xanthogenate als Derivate zur papierchromatographischen Trennung der Alkohole einige Nachteile mitbringt. Ihr Vorteil ist die schnelle Darstellung der Derivate, bequeme Sichtbarmachung und gute Trennung der ersten drei Homologen; bei den höheren Gliedern ist jedoch die Trennung in den bisher verwendeten Systemen nicht mehr so eindeutig. In einer vorangehenden Mitteilung[6] definierten wir die wichtigsten Eigenschaften, die die zur Chromatographie angewendeten Derivate besitzen müssen; da die Xanthogenate nicht stabil sind und ihre papierchromatographische Trennung Komplikationen mitbringt, erfüllen sie diese Forderungen nicht. Unseren Erfahrungen nach sind z.B. die 3,5-Dinitrobenzoesäureester vorteilhaftere Derivate, wie wir in einem ausführlichen Referat in dieser Zeitschrift zeigen wollen.

ZUSAMMENFASSUNG

Es wurde das Verhalten der homologen Alkylxanthogenate bei der Papierchromatographie studiert und einige Angaben früherer Autoren über die Reihenfolge der Verbindungen auf den Chromatogrammen berichtigt. Es wurde festgestellt, dass die Alkylxanthogenate in genügend alkalischen Lösungsmittelsystemen chromatographiert werden müssen um ihre Zersetzung zu vermeiden. Die Eignung der Xanthogenate als Derivate zur Papierchromatographie der Alkohole wird diskutiert.

SUMMARY

The behaviour of homologous alkyl xanthates in paper chromatography was studied and some information given by former authors concerning the sequence of the compounds on the chromatograms was considered. It was established that in order to avoid destruction of the alkyl xanthates these must be chromatographed in solvent systems that are sufficiently alkaline. The suitability of the xanthates as derivatives for the paper chromatography of alcohols is discussed.

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PAPER CHROMATOGRAPHY OF 2,4-DINITROPHENYL DERIVATIVES OF AMINES

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In work described elsewhere methods have been developed for the determination of amines occurring in small amounts in body fluids. These methods depended on preparation and spectrophotometric estimation of dinitrophenyl (DNP) derivatives of the amines. In the present paper are described methods for the paper chromatographic separation of DNP-amines.

EXPERIMENTAL

Preparation of dinitrophenyl derivatives of amines

For preparation of authentic specimens, the method of GRASSMANN, HÖRMAN AND ENDRES[1] was used, except for tryptamine. The results are summarised in Table I and Table II. DNP-tyramine (Table I) was recrystallised from its solution in ethanol–chloroform. The DNP-derivatives of amines listed in Table II were extracted with ethyl acetate and all, except DNP-β-mercaptoethylamine, were precipitated from solution in ethanol–chloroform. DNP-β-mercaptoethylamine was precipitated from its solution in monochlorobenzene.

DNP-tryptamine was prepared as follows: 30 mg of tryptamine hydrochloride were mixed with 50 ml of $NaHCO_3$–Na_2CO_3 buffer (approx. pH 10), and 1 ml of a solution of 1-fluoro-2,4-dinitrobenzene (3% v/v). The mixture was heated at 60° for 20 min and then 20 ml of 0.2 *N* NaOH in 60% dioxan were added. After heating for 1 h, the volume of the mixture was made to 200 ml with water and DNP-tryptamine was extracted with chloroform. The chloroform extract was evaporated to dryness and the residue was dissolved in ethanol and crystallised.

The method of isolation of DNP-derivatives of amines in body fluids is described elsewhere.

Preparation of reversed phase papers

(*a*) *Silicone-treated paper*[2]. A strip of Whatman No. 1 paper (17 × 56 cm) was placed in a shallow tray (18 × 60 cm), containing a 5% solution of silicone fluid (M.S. 1107) in cyclohexane. The solution was allowed to flow over the paper, by gently

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TABLE I

MONODINITROPHENYL DERIVATIVES OF AMINES

	Parent amine	*Nitrogen % of DNP-derivative*		*Melting point of DNP-derivative (°C)*		
		Calculated	*Found**	*Found*	*Literature*	*Reference*
1	Methylamine	21.3	21.3	175	176–177	3
					174.5–175	4
					176	5
2	Dimethylamine	19.9	19.35	74	78	6
					77	7
					87	8
					87	9
					87; 74	10
3	Ethylamine	19.9	20.52	115	113–114	9
4	Diethylamine	17.57	17.45	69	80	9
					80; 69	10
5	*n*-Propylamine	18.66	18.27	95	95	11
6	Isopropylamine	18.66	18.3	95	94–95	12
7	*n*-Butylamine	17.57	17.04	89	90	13
8	Isobutylamine	17.57	17.31	79	80	11
9	2-Methylbutylamine	16.6	16.50	49		
10	Isoamylamine	16.6	16.05	91	91	13
11	Ethanolamine	18.5	19.08	92	92	1
12	*β*-Phenylethylamine	14.6	14.34	155	154	14
13	Tyramine	13.86	13.1	156		
14	Pyrrolidine	17.72	17.65	102		
15	Piperidine	16.73	16.55	95		
16	Tryptamine	17.17	17.23	217		

* All nitrogen determinations were carried out by Messrs. WEILER AND STRAUSS, Microanalytical Laboratory, Oxford. *Comment*: Various melting points are recorded in the literature for DNP-dimethylamine[15] but there is no mention of dimorphism. BLANKSMA AND SCHREINEMACHERS[10] were the first to point out that DNP-dimethylamine and DNP-diethylamine can be obtained in two forms having different melting points.

TABLE II

BIS-DINITROPHENYL DERIVATIVES OF AMINES

	Parent amine	*Nitrogen % of DNP-derivative*		*Melting point of DNP-derivative*
		Calculated	*Found*	*Found*
17	Tetramethylenediamine (putrescine)	20.0	19.2	Shrinks and decomposes at 182°
18	Pentamethylenediamine (cadaverine)	19.35	18.68	173°
19	Agmatine	24.24	24.05	192°
20	Cysteamine (*β*-mercaptoethylamine)	17.11	16.45	188°
21	Histamine	22.12	21.48	Shrinks and decomposes at 195°

tilting the container from side to side. The procedure was carried out with both sides of the paper. The paper was then pressed between two sheets of absorbent paper and was then dried in an oven at 110° for 1 h.

(*b*) ***Paraffin-treated paper.*** A strip of Whatman No. 3 MM paper (17 × 56 cm) was placed in a shallow tray (18 × 60 cm) containing a 10% solution of liquid paraffin (B.P.) in petroleum ether (boiling range 40–60°). The same procedure as described in (a) was followed, and the paper was allowed to dry in air.

(*c*) *Acetylated paper*. Fully acetylated paper was prepared by the method of ZIJP[16]. Ten strips of Whatman No. 1 paper (16 × 56 cm) were loosely rolled and placed in a wide-mouthed glass vessel of 2 l capacity. Sufficient glacial acetic acid was poured into the vessel so as to cover the paper. After 4 h, the acetic acid was poured out as completely as possible. The acetylating mixture consisted of 350 ml of acetic anhydride, 450 ml of glacial acetic acid, 1000 ml of toluene and 0.8 ml of 60% perchloric acid. The mixture was cooled to about 10° and was then poured into the vessel. During the first three hours, the vessel was gently shaken, care being taken to keep the temperature below 20°. The next day the acetylating mixture was poured out and the papers were washed three times with water. The papers were then transferred to a water bath and were washed for 4 h in a flowing stream of water. Finally they were dried, first in air and then in an oven at 110° for 10 min.

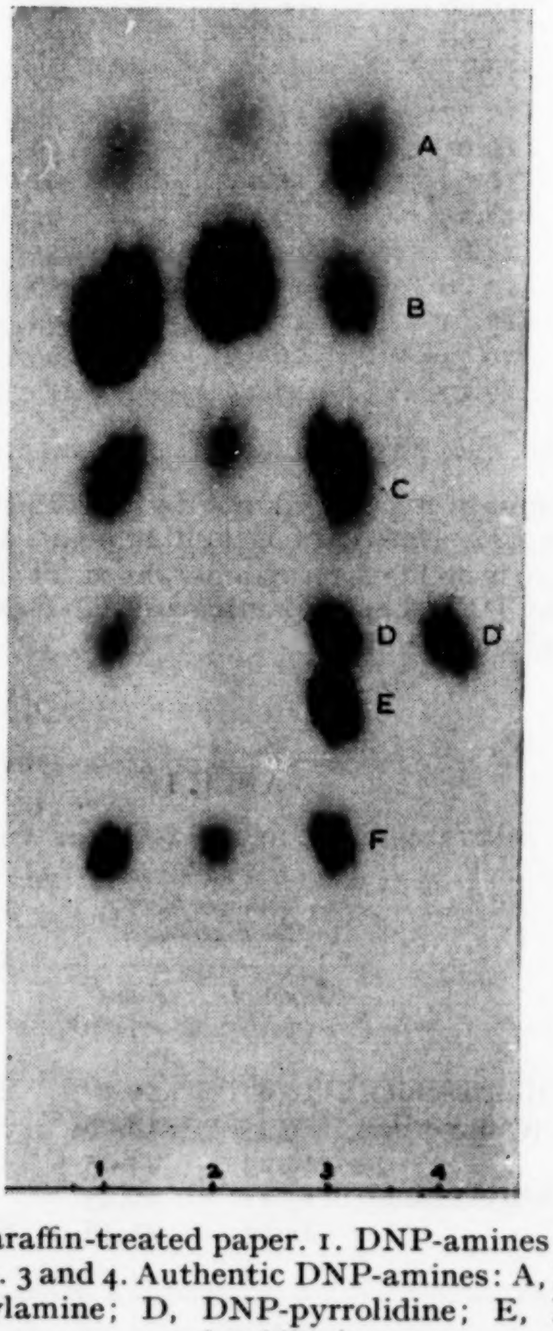

Fig. 1. Chromatography on paraffin-treated paper. 1. DNP-amines from normal urine. 2. DNP-amines from pathological urine. 3 and 4. Authentic DNP-amines: A, DNP-methylamine; B, DNP-dimethylamine; C, DNP-ethylamine; D, DNP-pyrrolidine; E, DNP-diethylamine; F, DNP-piperidine.

(*d*) *Benzoylated paper.* Benzoylated paper was prepared by the method described by MICHEEL AND ALBERS[17]. Benzoic anhydride and pyridine were mixed in the proportion of 1:5 and the mixture was cooled in ice-water. Ten strips of Whatman No. 1 paper (4 × 40 cm) were rolled together and inserted into a wide-necked Quickfit flask. Sufficient of the benzoylating mixture was poured into the flask so as to cover the paper and the mixture was refluxed at 95° for 8 h. At the end of this period, the strips were washed first with petroleum ether, then with water and finally with boiling methanol. They were dried in air.

Chromatography and location of DNP-amines

Chromatograms were run by the descending technique, the lower edge of the paper being serrated to allow even flow of the solvent from the paper. Using all types of reversed phase paper, the best solvent was found to be the upper phase of a mixture of chloroform, methanol and water, 10:10:6[2,18]. When paraffin-treated paper was used, the solvent was prepared by mixing chloroform, methanol, water and liquid paraffin, 10:10:6:4, and allowing the mixture to stand for at least 8 h, when the two phases become almost clear. In all cases, the lower phase was poured into a dish and left on the floor of the tank.

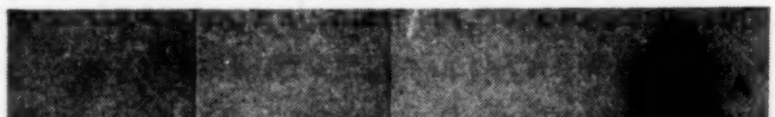

Fig. 2. Chromatography on benzoylated paper. A, DNP-ammonia; B, DNP-methylamine; C, DNP-dimethylamine; D, DNP-ethylamine; E, DNP-*n*-propylamine; F, DNP-diethylamine; G, DNP-piperidine; H, DNP-*n*-butylamine; I, DNP-isoamylamine; J, DNP-β-phenylethylamine.

In general chromatograms were run for 16 h (silicone-treated paper); 18 h (paraffin-treated paper); 24 h (acetylated paper) or 64 h (benzoylated paper).

DNP-amines were conveniently located by their characteristic appearance under u.v. light (Hanovia lamp, fitted with a Wood's glass filter). A permanent record could be obtained by direct photography of u.v.-illuminated chromatograms. Typical examples are shown in Figs. 1 and 2.

DISCUSSION

In preliminary experiments attempts were made to separate authentic DNP-amines on untreated Whatman No. 1 paper. The following solvents were used.

1. *tert.*-Amyl alcohol saturated with phthalate buffer pH 6 (BLACKBURN AND LOWTHER[19]).
2. Chloroform–2-propanol–0.05 *M* potassium benzoate, 45:49:6, v/v (MUNIER AND PÉNASSE[20]).
3. Cyclohexane–2-propanol–0.05 *M* potassium benzoate, 60:35:4, v/v (MUNIER AND PÉNASSE[20]).
4. Carbon tetrachloride–isopropanol–0.05 *M* potassium benzoate, 56:40:4, v/v (MUNIER AND PÉNASSE[20]).
5. Butanol–ethanol–ammonia, 40:10:50, v/v (KENT *et al.*[21]).
6. Decalin–acetic acid–isoamyl alcohol, 15:10:4, v/v (GRASSMAN *et al.*[1]).

In all cases the mixtures moved as a single spot at the solvent front. However, with butanol, acetic acid and water (4:1:5, PARTRIDGE[22]) fairly satisfactory resolution of the DNP-derivatives of tyramine, tryptamine, histamine and ethanolamine could be obtained.

Attention was therefore turned to using reversed phase paper. Four different types of paper were used.

(1) Silicone-treated paper[2,18].
(2) Paraffin-treated paper.
(3) Acetylated paper[23,17].
(4) Benzoylated paper[17].

In all the four systems used for the separation of DNP-amines, it was observed, both in the case of primary as well as secondary amines, that as the number of carbon atoms increases, there is a fall in the rate of movement on the chromatogram.

β-Phenylethylamine does not follow the common pattern. On silicone-treated paper as well as on paraffin-treated paper it runs between DNP-*n*-butylamine and DNP-isoamylamine, whereas on acetylated paper and on benzoylated paper it is slower than DNP-isoamylamine.

The separation of DNP-methylamine and DNP-dimethylamine is not satisfactory on silicone-treated paper, but good separation can be achieved by using paraffin-treated paper, acetylated paper or benzoylated paper.

Resolution of DNP-amines is best on benzoylated paper (Fig. 2). However, both acetylated paper and benzoylated paper take considerable time to prepare and they do not retain satisfactory properties over a long period.

For routine work, chromatography on paraffin-treated paper is the simplest and the most satisfactory procedure. Small differences in the amount of paraffin, incorporated into the paper, cause considerable variations in R_F values. However, fairly consistent results are obtained if rates of movement are measured relative to the rate of movement of DNP-methylamine. This is illustrated in Table III, where

$$R_{Me} = \frac{\text{Distance travelled by DNP-amine}}{\text{Distance travelled by DNP-methylamine}}$$

Mixtures of DNP-amines listed in Table III were applied to strips of paraffin-treated paper. In each column are shown the mean results of a group of three determinations using a single batch of paper, and the whole experiment was repeated on four occasions using different batches of paper.

TABLE III

CHROMATOGRAPHY ON PARAFFIN-TREATED PAPER

	R_{Me} *determined in each experiment*				*Mean* R_{Me}
	1	*2*	*3*	*4*	
DNP-methylamine	1	1	1	1	1
DNP-ethylamine	0.71	0.69	0.69	0.68	0.69
DNP-*n*-propylamine	0.48	0.46	0.46	0.45	0.46
DNP-isopropylamine	0.48	0.46	0.46	0.45	0.46
DNP-*n*-butylamine	0.33	0.31	0.31	0.30	0.31
DNP-isobutylamine	0.33	0.31	0.31	0.30	0.31
DNP-β-phenylethylamine	0.28	0.26	0.27	0.25	0.26
DNP-isoamylamine	0.22	0.19	0.21	0.20	0.20
DNP-dimethylamine	0.87	0.84	0.85	0.85	0.85
DNP-pyrrolidine	0.54	0.52	0.51	0.50	0.52
DNP-diethylamine	0.48	0.46	0.46	0.45	0.46
DNP-piperidine	0.32	0.31	0.31	0.30	0.31
DNP-2-methylbutylamine	0.22	0.20	0.21	0.19	0.20
DNP-ethanolamine	1.2	1.2	1.2	1.3	1.2
DNP-tyramine	1.2	1.2	1.2	1.3	1.2
DNP-histamine	1.1	1.1	1.1	1.1	1.1
DNP-tryptamine	0	0	0	0	0
DNP-agmatine	0	0	0	0	0
DNP-tetramethylenediamine	0	0	0	0	0
DNP-pentamethylenediamine	0	0	0	0	0
DNP-β-mercaptoethylamine	0	0	0	0	0

Results shown in Tables IV and V were obtained similarly. In the case of benzoylated paper, the results shown in Table VI are the mean of two readings and the experiment was repeated twice.

Amines in human urine

In pooled extracts of normal and pathological human urines the following amines were identified as the DNP-derivatives by comparison with authentic DNP-amines: methylamine, dimethylamine, ethylamine, pyrrolidine and piperidine (Fig. 1). The identity of the DNP-derivatives of the last two was further supported by their absorption spectra determined after elution. The amount of amines excreted by man is

TABLE IV

CHROMATOGRAPHY ON SILICONE-TREATED PAPER

	R_{Me} determined in each experiment				Mean R_{Me}
	1	*2*	*3*	*4*	
DNP-methylamine	1	1	1	1	1
DNP-ethylamine	0.79	0.83	0.79	0.78	0.80
DNP-*n*-propylamine	0.62	0.59	0.62	0.60	0.61
DNP-isopropylamine	0.62	0.59	0.62	0.60	0.61
DNP-*n*-butylamine	0.40	0.43	0.44	0.40	0.42
DNP-isobutylamine	0.40	0.43	0.44	0.40	0.42
DNP-β-phenylethylamine	0.33	0.36	0.34	0.33	0.34
DNP-isoamylamine	0.29	0.33	0.28	0.28	0.29
DNP-dimethylamine	0.92	0.94	0.91	0.91	0.92
DNP-pyrrolidine	0.64	0.70	0.65	0.64	0.66
DNP-diethylamine	0.54	0.58	0.54	0.54	0.55
DNP-piperidine	0.40	0.43	0.39	0.38	0.40
DNP-2-methylbutylamine	0.30	0.29	0.29	0.28	0.29
DNP-ethanolamine	1.1	1.2	1.1	1.1	1.1
DNP-tyramine	1.1	1.2	1.1	1.1	1.1
DNP-histamine	1.0	1.0	1.0	1.0	1.0
DNP-tryptamine	0	0	0	0	0
DNP-agmatine	0	0	0	0	0
DNP-tetramethylenediamine	0	0	0	0	0
DNP-pentamethylenediamine	0	0	0	0	0
DNP-β-mercaptoethylamine	0	0	0	0	0

TABLE V

CHROMATOGRAPHY ON ACETYLATED PAPER

	R_{Me} determined in each experiment				Mean R_{Me}
	1	*2*	*3*	*4*	
DNP-methylamine	1	1	1	1	1
DNP-ethylamine	0.78	0.75	0.75	0.79	0.77
DNP-*n*-propylamine	0.53	0.52	0.55	0.54	0.53
DNP-isopropylamine	0.54	0.52	0.55	0.54	0.53
DNP-*n*-butylamine	0.38	0.39	0.38	0.38	0.38
DNP-isobutylamine	0.38	0.39	0.38	0.38	0.38
DNP-β-phenylethylamine	0.16	0.17	0.15	0.16	0.16
DNP-isoamylamine	0.25	0.26	0.27	0.25	0.26
DNP-dimethylamine	0.89	0.85	0.85	0.87	0.86
DNP-pyrrolidine	0.50	0.49	0.46	0.48	0.48
DNP-diethylamine	0.50	0.49	0.46	0.46	0.48
DNP-piperidine	0.40	0.39	0.40	0.38	0.39
DNP-2-methylbutylamine	0.27	0.26	0.26	0.24	0.26
DNP-ethanolamine		runs off the paper			—
DNP-tyramine	0.46	0.46	0.50	0.48	0.47
DNP-histamine	0.15	0.19	0.16	0.18	0.17
DNP-tryptamine	0.1	0.1	0.08	0.09	0.09
DNP-agmatine	0.04	0.02	0.03	0.02	0.03
DNP-tetramethylenediamine	0.05	0.03	0.03	0.04	0.04
DNP-pentamethylenediamine	0.04	0.02	0.03	0.02	0.03
DNP-β-mercaptoethylamine	0.04	0.02	0.03	0.02	0.03

considerably less than the amount of ammonia excreted; but the ammonia which, if not removed, tends to dominate the chromatogram, can be eliminated by treatment with mercuric oxide[24], as described elsewhere. However, it may be mentioned that HgO-treatment causes considerable losses of ethanolamine, tyramine, β-phenylethylamine, tryptamine and histamine but leaves the aliphatic amines unaffected.

TABLE VI

CHROMATOGRAPHY ON BENZOYLATED PAPER

	R_{Me} determined in each experiment		*Mean R_{Me}*
	1	*2*	
DNP-methylamine	1	1	
DNP-ethylamine	0.74	0.71	0.73
DNP-*n*-propylamine	0.48	0.50	0.49
DNP-isopropylamine	0.48	0.50	0.49
DNP-*n*-butylamine	0.40	0.43	0.41
DNP-isobutylamine	0.40	0.43	0.41
DNP-β-phenylethylamine	0.18	0.16	0.17
DNP-isoamylamine	0.26	0.24	0.25
DNP-dimethylamine	0.90	0.89	0.89
DNP-pyrrolidine	0.47	0.50	0.48
DNP-diethylamine	0.55	0.58	0.56
DNP-piperidine	0.38	0.35	0.36
DNP-2-methylbutylamine	0.27	0.25	0.26
DNP-ethanolamine	runs off the paper		—
DNP-tyramine	0.41	0.38	0.39
DNP-histamine	0.12	0.10	0.11
DNP-tryptamine	0.12	0.10	0.11
DNP-agmatine	0.17	0.15	0.16
DNP-tetramethylenediamine	0.06	0.05	0.05
DNP-pentamethylenediamine	0.03	0.03	0.03
DNP-β-mercaptoethylamine	0.04	0.04	0.04

SUMMARY

The most satisfactory type of support for routine use in reversed phase paper chromatography of dinitrophenyl (DNP) derivatives of amines has been found to be paraffin-treated paper, which gives good resolution, is relatively easy to prepare and handle, is stable and on which the solvent, a mixture of chloroform, methanol and water, runs reasonably fast.

Human urine contains methylamine, dimethylamine, ethylamine, pyrrolidine and piperidine.

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CHLORANILIC ACID AS A REAGENT IN THE PAPER CHROMATOGRAPHY OF INORGANIC COMPOUNDS

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INTRODUCTION

Chloranilic acid (2,5-dichloro-3,6-dihydroxy-*p*-quinone) was first prepared by ERDMANN in 1854[1] and its structure determined by HANTZSCH AND SCHNITER[2, 3] and LING[4]. Its first analytical application was found by BARRETO[5, 6], who used it for the determination of calcium, strontium, barium and zinc. The calcium assay with chloranilic acid was further developed by TYNER[7], GAMMON AND FORBES[8], KAROLEFF[9], FROST-JONES AND YARDLEY[10], KUBO AND TSUTSUMI[11] and, more recently, by FLEURY, DESFOBERT AND EBERHARD[12]. VOIGT investigated the use of this reagent for the determination of zirconium[13] and BERTOLACINI AND BARNEY used its barium salt for the assay of minimum amounts of the sulfate ion[14, 15].

The present work was undertaken in order to determine the possibilities of using chloranilic acid as a reagent in inorganic paper chromatography, this first paper dealing with alkali and alkaline earth metals.

MATERIALS AND METHODS

Reagent

Chloranilic acid (HClA) was used as a 0.1 % solution in ethyl ether, and was kept in a dark bottle.

Sample solutions

Aqueous solutions of the acetates, containing 1 % of the cation were used. In some cases this concentration was reduced to 0.1 %.

Paper chromatography

This was carried out by the ascending technique, using Machery Nagel No. 261 filter paper and 96 % ethanol–2 N acetic acid (80:20) as solvent. The R_F values were comparable to those obtained when using Whatman No. 1 filter paper[16].

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Detection of the spots

The dipping technique gave better results than spraying. The thoroughly dried paper was placed in an enamel tray and covered with a thin layer of the HClA solution; after about 15 sec the reagent was returned to the bottle and the paper washed twice with ethyl ether. The chromatogram was then dried in a stream of hot air and observed under U.V. light.

Sensitivity evaluation

The first determinations were done by spotting increasing amounts (0.1–10 µg) of each cation on a strip of filter paper; the color was developed with the HClA reagent and observed with the naked eye and also under the U.V. lamp, in which case the sensitivity was about double. Under these conditions the spots were much more compact than after the chromatographic separation, when two to five times the same amount of material was needed. The results obtained in this way, however, were a good indication of the quantities necessary for paper chromatograms. In this case the same technique was used, and the weakest concentration applied to the paper was equal to the smallest one that gave a positive reaction in the first trial. The samples were spotted at 3 cm from the bottom of a 25 × 25 cm sheet of filter paper, chromatographed and developed as described before.

RESULTS

Chloranilic acid was found to be an extremely sensitive reagent for alkali and alkaline earth metals. Cations such as Na, K and Li were detected in amounts ranging from 0.2–1 µg after chromatographic separation. The sensitivity towards the alkaline earths was slightly smaller, ranging from 0.5–5 µg. The color of the reaction appears to be dependent on the amount of material in the spot. It is violet for small quantities of alkali metals, changing to brownish yellow with increasing concentrations. With 0.5 µg of sodium, for example, a brownish yellow spot with a violet halo is obtained

TABLE I

SENSITIVITY OF CHLORANILIC ACID TOWARDS ALKALI AND ALKALINE EARTH CATIONS IN SPOT TESTS AND ON PAPER CHROMATOGRAMS

Cation	*Sensitivity in µg*				
	Spot test		*Chromatogram*		
	daylight	*U.V.*	*daylight*	*U.V.*	R_F
Na^+	0.2	0.1	0.5	0.2	0.56
K^+	0.5	0.3	1.0	0.5	0.35
Li^+	0.4	0.2	1.0	0.5	0.89
Mg^{++}	2.0	1.0	10.0	5.0	0.85
Ca^{++}	2.0	1.0	5.0	2.0	0.70
Ba^{++}	1.0	0.4	1.0	0.5	0.14
Sr^{++}	2.0	1.0	2.0	1.0	0.26

after the chromatographic run. With alkaline earth cations the color varies from yellow to brownish yellow. In all cases, an absorption spot is seen under U.V. light.

Table I summarises the results obtained with the described technique.

DISCUSSION

The low specificity and high sensitivity of chloranilic acid towards a large number of cations, together with the simplicity of the method and the short time required, qualify HClA to be a very good reagent in paper chromatography. Positive reactions are obtained with small quantities of all the alkaline and alkali earth metals, and this is specially true in the first case (Na, K and Li), when its sensitivity is namely 25, 10 and 10 times better than the best usual detectors, such as aqueous cobaltinitrite, for example, when the detection limit is 5 μg[5]. Furthermore, the use of ether as solvent eliminates the risk of eluting material from the chromatographic spot.

ACKNOWLEDGEMENTS

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SUMMARY

The authors studied the use of chloranilic acid as a reagent in the paper chromatography of alkali and alkaline earth metals. Using a 0.1 % ether solution of the reagent and the dipping technique rather than spraying, they were able to detect 0.2–1 μg of Na, K and Li and 0.5–5 μg of Ba, Ca, Sr and Mg.

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PAPIERCHROMATOGRAPHIE EINIGER 2- UND 6-SUBSTITUIERTER PURINE

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Der Papierchromatographie der Purine wurde viel Aufmerksamkeit gewidmet insbesondere im Zusammenhang mit der Strukturuntersuchung und dem Vorkommen von Nucleinsäuren. Zum Nachweis dieser Substanzen dienen ihre bedeutende Absorption ultravioletten Lichts der Wellenlänge 250–260 mμ gegebenfalls bei Besprühen des Chromatogramms mit verschiedenen fluoreszierenden Substanzen, wie Fluorescein[1], Aesculin[2] u.a., sowie chemische Methoden. Von diesen seien die bekanntesten angeführt: Besprühen mit Silbernitrat und Schwefelwasserstoff nach CHARGAFF und Mitarb.[3], mit Silbernitrat und Kaliumchromat nach REGUERA UND ASIMOV[4], mit Sublimat und Eosin nach MICHL[5], mit Silbernitrat und Bromphenolblau nach WOOD[6], mit Kaliumpermanganat, Chlor und Kaliumhydroxyd nach GERLACH UND DÖRING[7], mit Kupfersulfat, Ammoniak und Diphenylcarbazid nach BLAZSEK[8]. Hiervon ist keines der erwähnten Nachweisreagenzien als selektiv für auf dem Purinskelett begründete Substanzen zu betrachten.

Von Lösungsmittelsystemen wurden für diese Substanzen zahlreiche geläufige Systeme beschrieben, die für Substanzen von hydrophilem Charakter geeignet sind: Grösstenteils wird die Grundlage dieser Systeme von niedrigeren aliphatischen Alkoholen im Gemisch mit Säuren (Chlorwasserstoffsäure, Essigsäure, Ameisensäure), Basen (Ammoniak) oder Puffern gebildet. Diese Systeme sind in vielen Übersichtsreferaten und in Büchern zusammenfassend dargestellt (siehe z.B.[9–14]).

Ausser den in biologischem Material vorkommenden Purinen wird in letzter Zeit einigen substituierten Purinen in Zusammenhang mit der Chemotherapie der Krebskrankheit erhöhte Aufmerksamkeit gewidmet. Die meisten 2-, 6- und 8-substituierten Purine wurden papierchromatographisch von TESHIMA und Mitarb.[15] untersucht, die zur Entwicklung das System Isopropanol–2 N Chlorwasserstoffsäure (65:35) verwendeten. ALBERT UND BROWN[16] entwickelten mit dem System n-Butanol–5 M Essigsäure (2:1) und BENDICH und Mitarb.[17] verwendeten verschiedene Systeme mit n-Butanol, *tert.*-Butanol, Isopropanol, Äthanol oder wässrige Ammoniumchloridlösung.

Bei unseren Untersuchungen bewerteten wir ausser einigen bereits chromatographierten Derivaten grösstenteils neu dargestellte Substanzen, die durch einen wenig hydrophilen Rest substituiert sind, so dass wir für sie andere Systeme verwenden mussten. Daneben untersuchten wir alle diese Substanzen auf ihre Reaktivität mit verschiedenen Nachweisreagenzien.

EXPERIMENTELLER TEIL

Papier

Alle Nachweisreaktionen und Entwicklung in wasserhaltigen Systemen erfolgten auf Papier Whatman No. 2. Chromatographie im System mit verankertem Formamid wurde auf Papier Whatman No. 4 durchgeführt.

Nachweis

Die Sichtbarmachung der einzelnen Substanzen wurde in Tüpfelreaktionen bei Auftragen von 1, 10 und 100 μg, in Zweifelsfällen noch nach chromatographischer Trennung in Systemen S 2 oder S 3 untersucht. Starke Reaktion wurde in der Tabelle I als +++ bezeichnet (Empfindlichkeit 1–3 μg), mittelstarke Reaktion als ++ (Empfindlichkeit ungefähr 10 μg), schwache Reaktion +, sehr schwache Reaktion —.

D 1. Beobachtung der Fluoreszenz um U.V.-Licht der Hochdruck-Quecksilberlampe (Philora).

D 2. Beobachtung von Fluoreszenz oder Verlöschen im Licht der Niederdruck-Queksilberlampe (Chromatolite).

D 3. Beobachtung des Verlöschens im Licht der Niederdruck-Quecksilberlampe nach Besprühen des Chromatogramms mit 0.0025 %iger Fluoresceinlösung in 0.5 *N* Ammoniak.

D 4. Das Chromatogramm wird in eine 2 %ige $AgNO_3$-Lösung eingetaucht, 5 min an der Luft trocknen gelassen und dann in eine 0.5 %ige $Na_2Cr_2O_7$-Lösung eingetaucht. Das überschüssige Reagens wurde aus dem Hintergrund mit dem 0.5 *N* HNO_3 herausgewaschen.

D 5. Das Chromatogramm wird auf 2 min in die Lösung von 20 mg Eosin und 3.25 g $HgCl_2$ in 10 ml Äthanol eingetaucht. Das überschüssige Reagens wird sodann mit 50 %igem wässrigen Äthanol ausgewaschen.

D 6. Das Chromatogramm wird durch eine, durch Vermischen gleicher Teile 0.4 %iger Lösung von Bromphenolblau in Aceton und 2 %iger Lösung von $AgNO_3$ hergestellte Lösung gezogen. Das überschüssige Reagens wird nach Trocknen des Chromatogramms an der Luft mit Wasser gewaschen.

D 7. Dragendorff-Reagens (D 144a[8]).

D 8. Jod-Azid-Reagens (D 163b[8]).

Lösungsmittelsysteme

Die Chromatogramme wurden absteigend bei der konstanten Temperatur von 20° in den folgenden Systemen entwickelt:

S 1. *n*-Butanol mit Wasser gesättigt + 5 % Formamid.

S 2. *n*-Butanol–Essigsäure–Wasser (4:1:5).

TABELLE

R_F-WERTE EINIGER

Purinderivat	Nachweisreaktionen*				
	D 1	*D 2*	*D 3*	*D 4*	*D 5*
6-Chlor-	G++	(+)	++	++	+
6-Hydroxy- (Hypoxanthin)	B+	+++	+++	+++	++
6-Amino- (Adenin)	o	+++	+++	+++	+++
2-Amino-6-hydroxy- (Guanin)	o	++	+++	++	+++
2-Methyl-6-hydroxy-	o	+++	+++	+++	o
6-Cyclohexylamino-	o	++	+++	+++	++
N,N′-Dipuryl-N,N′-di(chloräthyl)piperaziniumdichlorid	o	+++	+	+	o
6-Mercapto-	G++	o	++	G	+++
2-Mercapto-6-hydroxy-	B++	++	+++	G	++
2-Hydroxy-6-mercapto-	B+++	(B)	+++	G	+++
2-Methyl-6-mercapto-	G++	o	++	G	++
6-Carboxymethylthio-	o	o	+	+++	+
6-Carboxyäthylthio-	o	(G)	++	+++	++
6-Carboxypropylthio-	o	(G)	+++	+++	+++
6-Carboxybutylthio-	o	(G)	++	+	++
6-Carboxypentylthio-	o	(G)	++	+	++
6-Carboxyhexylthio-	o	(G)	+	+	+
2-Amino-6-carboxybutylthio-	B+++	(B)	(+++)	++	+++
2-Carboxybutylthio-6-hydroxy-	o	++	++	+++	+++
6-Carbäthoxypropylthio-	o	o	++	G	+
6-Carbäthoxybutylthio-	o	o	++	G	+
6-Carbäthoxyhexylthio-	o	o	+	G	+
6-Carboxybutylthio-, *β*-Cyclopentyläthylester	o	o	+	G	—
6-Carboxybutylthio-, Cyclohexylester	o	o	+	G	+
6-Carbbutoxybutylthio-	o	o	+	G	—
2-Hydroxy-6-carbäthoxybutylthio-	B++	(++)	(++)	+++	++
2-Hydroxy-6-carbbutoxybutylthio-	B++	(++)	(++)	++	++
2-Amino-6-carbäthoxybutylthio-	B++	(+++)	(++)	G	+++
6-Cyanpropylthio-	o	o	++	G	—
6-Carbamidopropylthio-	o	o	++	++	++
6-Carboxybutylthio-, *β*-Diäthylaminoäthylester	o	o	++	+	++ +

* B = Blaufärbung, G = Gelbfärbung, Werte in Klammern = Fluoreszenz anstatt Verlöschen.

S 3. *n*-Butanol gesättigt mit 1.5 *N* Ammoniak.

S 4. Isopropanol–Ammoniak–Wasser (10:1:1).

S 5. Papier imprägniert mit 50% äthanolischer Formamid; Chloroform fliesst durch.

S 6. Formamid/Benzol.

ERGEBNISSE

Die Ergebnisse sind am besten aus der Tabelle I ersichtlich. Aus dieser Tabelle geht hervor, dass der allgemeinste, allerdings nicht selektive Nachweis für Purine das Verlöschen dieser Substanzen nach Besprühen mit Fluorescein im Licht der Nieder-

I

PURINDERIVATE

			$R_F \cdot 100$					
D 6	D 7	D 8	S 1	S 2	S 3	S 4	S 5	S 6
+++		o	62	79	43	74	06	
+++		o	26	41	15	31		
+++		o	41	52	40	53		
+		o	16	L**	L	L		
+++	o	o	39	52	28	41	01	
+++		+	91L	92	89	89	63	
+++		+++	40	46	46	57	00	
+++	+++		38Z***	49	16Z	34Z		
+++	++		16Z	43	05Z	24Z		
+++	+++		25	45	06Z	23Z		
+++	+++	o	50	60	22	37	02	
—	o	o	06	66	04	23		
—	o	o	14L	79	06	24		
++	+	o	31L	80	08	26	00	
+	o	o	36L	88	10	29	01	
+	o	o	47L	89	16	36	03	
+	o	o	58L	92	25	43	07	
—	o	o	22L	80	11	30	00	
—	o	o	20L	76	03	12	00	
+++	+		80L	91	90	83	81	13
+++	o		80L	91	90	84	88	22
+++	o	+†	84L	92	90	85	92	58
+++	o	+†	90	93	88	88	93	84
+++	o	+†	90	93	88	87	94	71
+++	o	+†	90	93	88	85	93	60
++	+		67L	86	53	64	08	00
++	o		77L	89	65	75	33	00
+++	o	+†	75L	88	87	86	64	03
+++	o		65	83	61	71	32	01
+++	o		47	69	35	50	00	00
+++	o	+++	59L	70	86	82	07	00

** L = langgezogene Flecken. *** Z = Zersetzung.
† Atypische Reaktion für D 8. Weitere Erläuterungen siehe im EXPERIMENTELLEN TEIL.

druck-Quecksilberlampe ist. Diese Nachweisreaktion ist verhältnismässig empfindlich mit Ausnahme einiger in 6-Stellung durch eine längere aliphatische Kette substituierter Purine. Auch in diesen Fällen war der Nachweis empfindlicher, wenn das entsprechende Purin gleichzeitig in Stellung 2 substituiert war. Ohne Besprühen mit Fluorescein kamen im Licht der Niederdrucklampe nur nichtalkylierte durch einen einfachen Substituenten substituierte Purine zur Geltung.

Im U.V.-Licht der Hochdruck-Quecksilberlampe kamen die meisten der untersuchten Purine nicht zur Geltung. Eine Ausnahme bildeten unter den monosubstituierten Purinen 6-Chlorpurin und 6-Mercaptopurin. Sehr stark fluoreszierten Substanzen, die gleichzeitig in Stellung 2 und 6 substituiert waren, soweit zumindest eine Gruppe Schwefel enthielt.

Von den chemischen Reagenzien hat sich in unseren Versuchen am besten Bromphenolblau mit $AgNO_3$ (D 6) bewährt: Die meisten Substanzen reagierten bereits in der Menge von 1–10 μg. Schwächer reagierten nur durch eine Carboxyalkylthiogruppe substituierte Purine. Zu analogen Ergebnissen führte Sichtbarmachung durch Eosin mit $HgCl_2$ (D 5) mit dem Unterschied, dass die Empfindlichkeit mit Rücksicht auf die Rosafärbung schwächer ist. Das Reagens D 5 reagierte jedoch mit Carboxyalkylthiopurinen einigermassen stärker als das Reagens D 6, während D 6 wiederum mit Carbalkoxyalkylthiopurinen stärkere Reaktion ergab. Für eine Reihe von Purinen war das Reagens $AgNO_3 + Na_2Cr_2O_7$ (D 4) sehr empfindlich. Von den für eine bestimmte Gruppe spezifischen Reagenzien benützten wir das Jod-Azid-Reagens (D 8) für Substanzen mit einer freien Mercaptogruppe; Substanzen mit substituierter Mercaptogruppe reagierten mit diesem Reagens nicht. Substanzen, die im Molekül tertiären Stickstoff enthalten, wurden mit Dragendorff-Reagens (D 7) sichtbargemacht.

Bei der Wahl der Lösungsmittelsysteme konnten wir die untersuchten Purine im allgemeinen in zwei Gruppen einteilen: Derivate, bei denen bei der Chromatographie der hydrophile Substituent zur Geltung kam, und ferner Derivate, wo der hydrophobe Substituent überwog. Für die erste Gruppe liessen sich einige der für Purine geläufig verwendeten Systeme anwenden. Dabei bewährte sich am besten das saure System S 2, und für durch eine Carboxyalkylthiogruppe substituierte Purine das System S 3 oder S 4. Diese basischen Systeme waren für die meisten Substanzen mit freier Mercaptogruppe nicht verwendbar, weil in diesen Fällen Zersetzung auf dem Papier eintrat. Für die zweite Gruppe (namentlich Carbalkoxyalkylthiopurine) bewährten sich am besten die Systeme mit verankertem Formamid S 5 oder S 6.

Bei Beobachtung des Einflusses der einzelnen funktionellen Gruppen auf die R_F-Werte wurden die an anderen Stoffgruppen ermittelten Gesetzmässigkeiten bei substituierten Purinen bestätigt: Von den hydrophilen Gruppen setzten die R_F-Werte am meisten Carboxyl und Hydroxyl herab, etwas weniger die Mercaptogruppe, noch weniger die Aminogruppe. Bei der Carboxyl- und Aminogruppe spielte allerdings auch der pH-Wert des Systems eine wichtige Rolle. Komplizierte Verhältnisse lagen allerdings in den geläufigen Butanol-Systemen bei disubstituierten Derivaten vor, wo weitere Substitution nicht immer zu Werten führte, die nach der Regel über die Additivität der funktionellen Gruppen zu erwarten sind: So führte z.B. Substitution des Hypoxanthins durch eine weitere stark hydrophile Mercaptogruppe nicht zu einem bedeutenden Absinken des R_F-Wertes, sondern im Gegenteil zu schwacher Steigerung von 0.41 auf 0.43. Diese Anomalien in wässrigen Systemen verschwanden auch in Systemen mit verankertem Formamid, wo die Regel über die Additivität der funktionellen Gruppen ihre Bestätigung fand.

DANK

Alle in der Tabelle I aufgeführten Substanzen wurden in unserem Institut von Herrn Dr. SEMONSKÝ und Dr. ČERNÝ dargestellt, denen wir für die freundliche Überlassung dieser Proben danken.

ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurde das chromatographische Verhalten von einunddreissig 2- und 6-substituierten Purinen in sechs Lösungsmittelsystemen und ihr Nachweis mit acht Reagenzien beschrieben.

SUMMARY

The chromatographic behaviour of thirty-one 2- and 6-substituted purines in six solvent systems is described as well as their detection with eight reagents.

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ULTRAVIOLET FLUORESCENCE FOR DETECTION OF ORGANIC COMPOUNDS ON PAPER

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A procedure was developed earlier by the author for detecting phosphorylated sugars and other phosphate compounds on paper chromatograms utilizing the background ultraviolet fluorescence of quinine sulfate[1]. As little as 0.007 μmole of phosphorus could be detected. It has now been found that some modifications and extensions of the fluorescence procedure make it more generally applicable. The ultraviolet fluorescing compounds quinine sulfate and sulfosalicylic acid have been successfully applied for the detection of a wide variety of organic compounds. In some cases this universal detection procedure offers increases in sensitivity over classical methods.

In this study the fluorescing compound is used for the detection of compounds which, with only a few exceptions, did not naturally fluoresce or quench on the paper when viewed with the ultraviolet light source prior to the dip procedure. Nothing is known concerning the mechanism by which the compounds interfere with the fluorescence of either the quinine sulfate or sulfosalicylic acid.

MATERIALS AND METHODS

The organic compounds tested here were chromatographed on the papers by an appropriate solvent system. The area occupied by the organic compound was detected by a simple procedure in which the developed and dried chromatogram was dipped in a solution of 0.5% quinine sulfate$\cdot 2H_2O$ in absolute ethanol or in a freshly prepared solution of 1.0% sulfosalicylic acid in acetone. In the case of some O-methyl sugars 1% quinine sulfate in absolute ethanol was used as is shown in Table I. The papers were dried at room temperature and the spots were detected by viewing the papers under ultraviolet light in a darkened room. The organic compounds are usually observed as light fluorescent areas against an intense gray fluorescent background.

Two ultraviolet light sources were used in these studies. A low-pressure mercury source, filtered to exclude visible light, produced predominantly ultraviolet radiation in the 2537 Å unit region. A long-wave lamp with ultraviolet radiation in the 3600 Å unit region and also filtered to exclude visible light was used as an alternate ultraviolet source.

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* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

The dip procedure has worked well with a variety of papers including Whatman No. 1 and Schleicher & Schuell No. 507*. In almost every case prior washing of the paper was found to be beneficial since interfering material from the paper is often present.

RESULTS AND DISCUSSION

The sensitivity of the dip procedure for spot detection is shown in Table I. The sapogenin lucernic acid was detected by this procedure on paper chromatograms. No other procedures have been successful for the detection of lucernic acid. The positions of other sapogenins and the saponins, with the exception of lucernic acid, were verified by the use of a Lieberman-Burchard dip procedure[2].

Papers used for sugar analyses should be dipped immediately after development and drying of the chromatograms. Although results were variable, in some cases marked increases in sensitivity over that reported in this paper were obtained with sugars if the paper was dipped in a solvent containing equal volumes of 1% quinine sulfate·$2H_2O$ and absolute ethanol saturated with boric acid. This is the only instance where the addition of an inorganic compound or high boiling point organic material to the detection dip or developing solvent was not deleterious. For example, losses in sensitivity because of a masking of the paper occurred in the presence of ferric chloride, ammonium sulfate, ammonium acetate, sodium phosphates, potassium phosphates, phosphoric acid, sulfuric acid and phenol. However, a molybdic acid solution containing quinine sulfate was reported for the detection of orthophosphate on paper[3], but in this case the background fluorescence of the paper was apparently used to amplify the phosphomolybdate spot.

It seems likely that many organic compounds other than those tested here could be detected by this procedure. In addition other fluorescing materials besides quinine sulfate and sulfosalicylic acid should be suitable for use as detection reagents for this procedure.

The dip procedure, in addition to the ease of use, offers the advantage, at least in the case of quinine sulfate, of employing a non-reacting spot-detection compound. If the spots of organic material to be detected are not soluble in alcohol or acetone, the quinine sulfate or sulfosalicylic acid can be washed or chromatographed off the paper with ethanol or acetone, thus leaving the spots free for elution and further determinations. If, however, the material of the spot to be detected is soluble in alcohol or acetone, the dipping of the paper should be done as rapidly as possible and the paper should be immediately hung to drain in the same direction in which the spots were developed.

In only a few cases did the sulfosalicylic acid or quinine sulfate, even when left on the paper, interfere with other spot-detection procedures. This lack of interference is advantageous. Following the marking of the spots under a minimum ultraviolet light exposure, it is possible to run specific reducing sugar tests, ketose tests, ninhydrin

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* Mention of specific products does not imply recommendation by the Department of Agriculture over others of a similar nature not mentioned.

TABLE I

ORGANIC COMPOUNDS DETECTED BY BACKGROUND FLUORESCENCE ON PAPER CHROMATOGRAMS

Dip A: 0.5% quinine sulfate$\cdot 2H_2O$ in absolute ethanol. Dip B: 1.0% quinine sulfate$\cdot 2H_2O$ in absolute ethanol. Dip C: 1% sulfosalicylic acid in acetone.

Compound tested	*Minimum amount of compound detectable, in μg*					
	Dip A		*Dip B*		*Dip C*	
	2537 Å	*3600 Å*	*2537 Å*	*3600 Å*	*2537 Å*	*3600 Å*
Betaine	20	20			20	20
Thiamine	10	10			5	5
Fructose	75	50			20	50
Dextrose	75	50			20	20
Mannose	75	50			20	50
Galactose	75	50			50	50
Sucrose	75	50			20	50
Raffinose	75	50			20	50
Inositol	75	75			20	50
Sorbitol	50	50			20	50
Glucuronic acid	50	50			50	75
Dimethyl-glucose			50	50	50	> 100
Tetramethyl-methyl-mannoside			50	50	50	100
2,3,4-Trimethyl-xylitol			100	100	50	50
Trimethyl-arabofuranoside			50	50	50	50
2,3,4,6-Tetramethyl-glucose			50	50	50	50
Hydroxyproline	25	25			> 40	15
Valine	> 40	> 40			> 40	15
Tryptophan	15	15			5	5
Methionine	> 40	> 40			> 40	15
Glutamic acid	15	15			> 40	15
Threonine	> 40	> 40			> 40	15
Cystine	15	> 40			5	> 40
Tyrosine	5	5			15	15
Oleanolic acid	50	50			> 100	> 100
Soyasapogenol B	100	100			> 100	> 100
Soyasapogenol C	50	50			> 100	> 100
Lucernic acid	20	20			> 100	> 100
Strawberry clover saponin	20	20			> 100	> 100
Hederin	10	10			> 100	> 100
Ladino clover saponin	10	10			50	50

procedures, etc. Quinine sulfate had no effect on subsequent ninhydrin procedures. When sulfosalicylic acid remained on the papers, however, the ninhydrin reaction resulted in a cherry-red color for valine and threonine, pink for methionine and blue for cystine.

It was noted throughout these studies but especially with the saponins and sapogenins that the papers must be developed long enough to move the spots under study away from contaminating organic and inorganic materials. In some cases where an excess of impurities was present, the material spotted was observed only as a large streaking of poorly defined contaminating substances present in amounts too large to separate into discrete spots.

SUMMARY

A general fluorescence procedure utilizing 0.5% and 1% solutions of quinine sulfate·$2H_2O$ in absolute ethanol or 1% sulfosalicylic acid in acetone has been developed for the non-selective detection of a variety of organic compounds on paper chromatograms. When the developed and dried papers are dipped in the appropriate solution, dried and observed under ultraviolet light, organic compounds are visible against the background fluorescence of the paper. Inorganic or non-volatile developing solvents interfere with this procedure.

Included in the list of compounds which were tested is the sapogenin lucernic acid which has heretofore defied detection on paper chromatograms.

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[2] G. R. Van Atta and J. Guggolz, *J. Agr. Food Chem.*, 6 (1958) 849.
[3] L. Velluz and M. Pesez, *Bull. soc. chim. France*, 17 (1950) 868.

Short Communications

The effect of the surface area on the separation in gas–liquid partition chromatography*

During the evaluation of a new instrument** for the determination of specific surface areas, the surface area of five chromatographic support materials were measured. These data led us to investigate the difference in the separation effect of chromatographic columns using these support materials.

The five support materials used for the investigations were the following:

Firebrick, 60/100 mesh (Johns-Manville)
Chromosorb, 60/100 mesh (Johns-Manville)
Chromosorb W, Type 6, 60/100 mesh (Johns-Manville)
Celite 545, 60/100 mesh (Johns-Manville)
Teflon-1, < 35 mesh (du Pont)

The specific surface area were measured using the method described by NELSEN AND EGGERTSEN[1] and by LEE AND STROSS[2]. The results are as follows:

Firebrick	4.14 m^2/g
Chromosorb	2.86 m^2/g
Chromosorb W	1.41 m^2/g
Celite	1.14 m^2/g
Teflon	0.23 m^2/g

To investigate the separation effect using these support materials, we prepared four 2 m long columns containing Ucon Oil 550-X (Union Carbide Chemical Corp.) as stationary phase. The amount of liquid on the column material was 28.5 wt.% excepting for the Teflon column where the column material contained 16.5 wt.% liquid phase.

Two test mixtures were used for the investigations: the Phillips No. 38 hydrocarbon mixture as a typical non-polar sample and an alcohol mixture as a typical polar sample. The concentration of the mixtures was as follows:

Phillips No. 38	
1. Propane	10 vol.%
2. Isobutane	15 vol.%
3. *n*-Butane	30 vol.%
4. Isopentane	15 vol.%
5. *n*-Pentane	30 vol.%

Alcohol mixture	
1. Methyl alcohol	10 vol.%
2. Ethyl alcohol	10 vol.%
3. Isopropyl alcohol	15 vol.%
4. *sec.*-Butyl alcohol	25 vol.%
5. Isobutyl alcohol	20 vol.%
6. *n*-Butyl alcohol	20 vol.%

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* Part of a paper presented at the 11th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Pittsburgh, Pa. (U.S.A.), March 1, 1960.

** The Perkin-Elmer – Shell Model 212 Sorptometer.

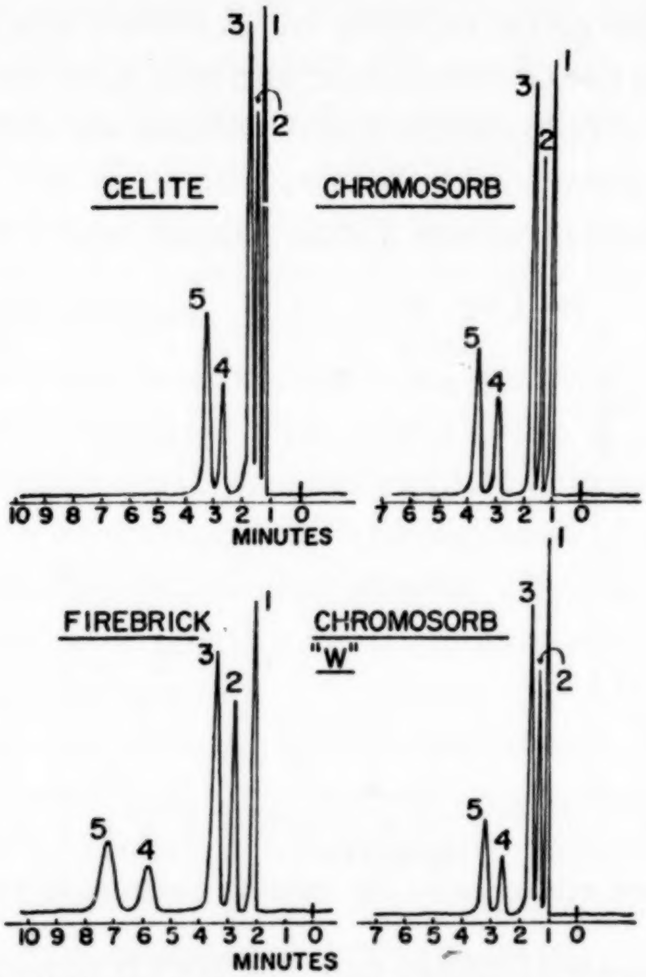

Fig. 1. Analysis of the Phillips No. 38 mixture. Temperature: 35°.

The resulting chromatograms are shown in the figures. The separation for *hydrocarbons* (Fig. 1) on all four columns with diatomaceous earth derivatives as support material was good. Although materials with the same mesh size were used, Firebrick had a much higher flow resistance; therefore, the carrier gas flow was always considerably lower (even with much higher inlet pressure) than in the other three analyses. For *alcohols* (Fig. 2) Chromosorb W and Celite showed the best separation;

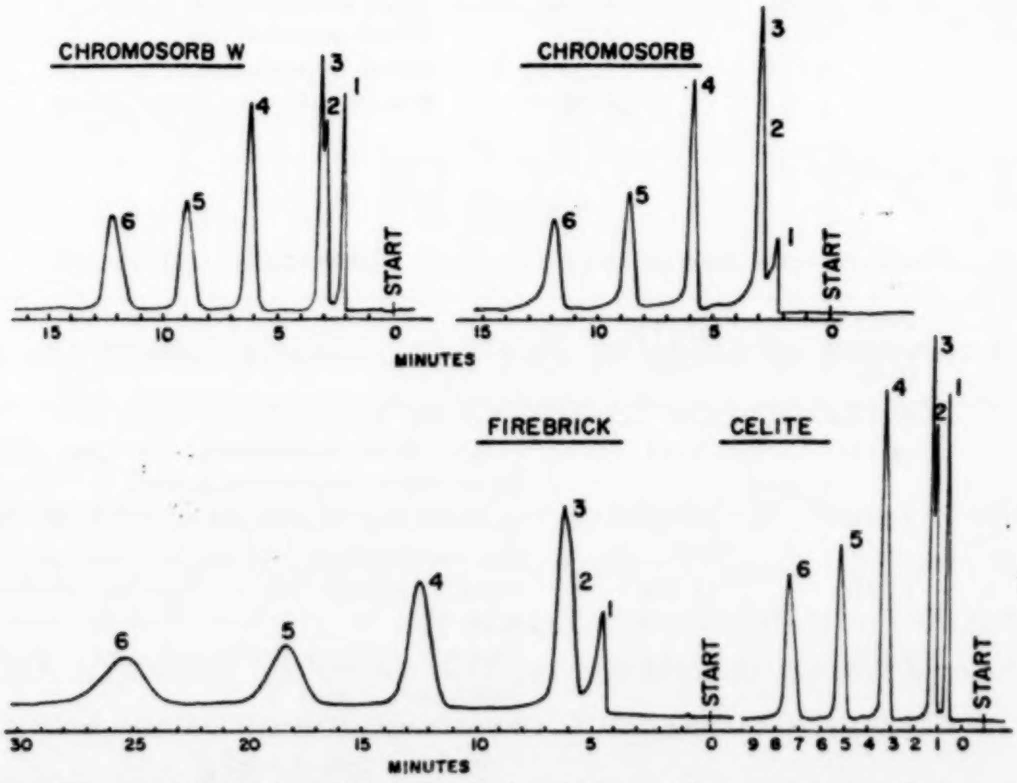

Fig. 2. Analysis of the alcohol mixture. Temperature: 85°.

according to an equation given recently by KAISER[3], the separation effect of the peaks 2 and 3 is 37.9 % on the Celite column, and 30.0 % on the Chromosorb W column. The separation of hydrocarbons on the *Teflon* column was extremely poor; that of the alcohols on this column considerably better, with even the first peak symmetrical; however, the overall efficiency of the Teflon column was still lower than that of the other columns (Fig. 3).

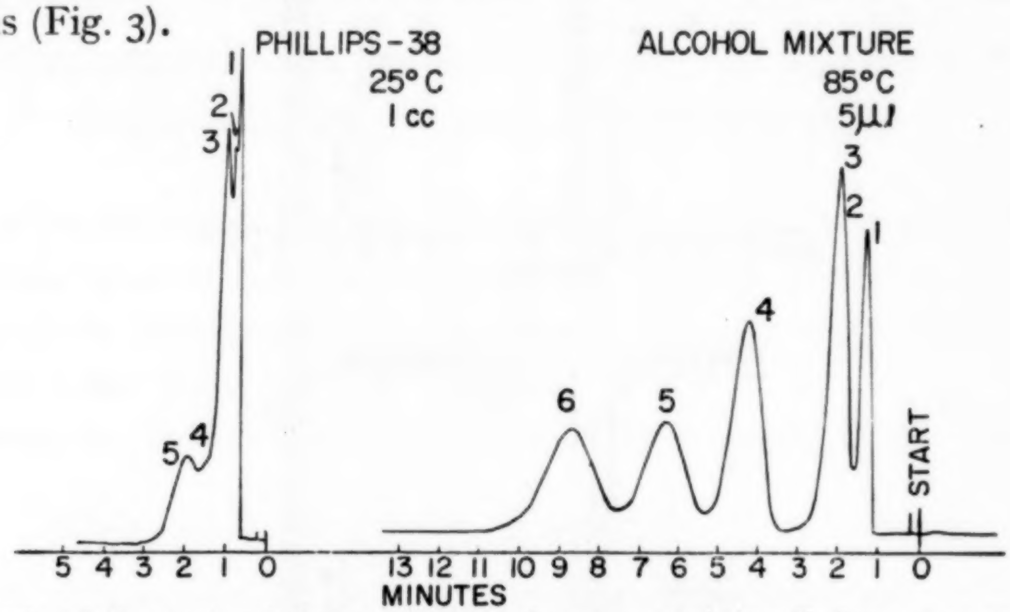

Fig. 3. Analysis of the test mixtures on the column containing Teflon as support material.

The number of theoretical plates and the HETP values were calculated for the last peaks according to the recommendation of the London symposium[4]. The results of the calculation are summarized in Tables I and II. As shown, the theoretical plate

TABLE I

COMPARATIVE MEASUREMENTS WITH THE HYDROCARBON MIXTURE

Support material	*Number of theoretical plates*	*HETP mm*	*Remark*
Firebrick	827	2.42	High flow-resistance. Good separation
Chromosorb	1752	1.14	Good separation
Chromosorb W	1218	1.64	Good separation
Celite	1138	1.71	Good separation
Teflon	43	46.85	Practically no separation

TABLE II

COMPARATIVE MEASUREMENTS WITH THE ALCOHOL MIXTURE

Support material	*Number of theoretical plates*	*HETP mm*	*Remark*
Firebrick	827	2.41	High flow-resistance. Strong tailing for 1. No separation between 2 and 3
Chromosorb	1985	1.01	Tailing for each peak, very strong tailing for 1. No separation between 2 and 3
Chromosorb W	1726	1.16	Small tailing for 1. Partial separation between 2 and 3
Celite	1486	1.34	Sharp symmetrical peaks. Partial separation between 2 and 3
Teflon	207	9.66	Symmetrical peaks. Poor separation. No separation between 2 and 3

numbers for the diatomaceous earth columns are similar, and even if they are lower, the separation shown on the chromatogram was good. The Teflon column, however, showed a very poor separation for the hydrocarbons, and a better, but not sufficient separation for the alcohols.

Five conclusions can be drawn from these data:

1. In general, the use of Chromosorb, Celite and Chromosorb W as support material results in a similar separation effect.

2. The use of Firebrick has no advantages; it has a very high flow resistance and at the same time, no better separation effect than Celite or Chromosorb.

3. In analyzing polar compounds, the tailing (particularly for the first components of the homologous series) is considerable with Chromosorb and Firebrick, even if, as in the case of Chromosorb, the theoretical plate number calculated for higher homologues is better. At the same time, this polarity effect diminishes the separation among the first members of the homologous series.

4. The specific surface area of the support material does not influence particularly the separation effect of the column if the surface area is over a certain limit, and in this case the influence of the polarity is considerably higher. This limit seems to be according to our data at about 1 m^2/g, which is contradictory to other data[5] where a minimum necessary surface area of about 3 m^2/g is given.

5. The use of Teflon as support material is only preferable where the extremely high polarity of the sample component(s) makes it necessary.

The Perkin-Elmer Corporation, Norwalk, Conn. (U.S.A.) L. S. ETTRE

[1] F. M. NELSEN AND F. T. EGGERTSEN, *Anal, Chem.*, 30 (1958) 1387.
[2] C. F. LEE AND F. H. STROSS, *Division of Analytical Chemistry*, 135th National American Chemical Society Meeting, Boston, Mass., U.S.A., April 9, 1959.
[3] R. KAISER, *Gas Chromatographie*, Akad. Verlagsgesellschaft Geest & Portig K.G., Leipzig, 1960, p. 33.
[4] D. H. DESTY (Editor), *Vapour Phase Chromatography*, Butterworths, London, 1957, p. xii.
[5] R. KAISER, *Gas Chromatographie*, Akad. Verlagsgesellschaft Geest & Portig K.G., Leipzig, 1960, p. 39.

Received March 23rd, 1960

J. Chromatog., 4 (1960) 166–169

Gas chromatographic analysis of volatile components in the presence of excess of non-volatiles

The analysis of dissolved gases in petroleum fractions has been the subject of some recent publications[1–3]. The fundamental principle of these methods is the same: a large (up to 1 ml) liquid sample is introduced into the chromatograph; the heavier hydrocarbons are adsorbed on a trap column installed in series before the chromatographic column and the dissolved gases are analyzed on the latter. The functional schematic of such a unit is given in Fig. 1.

J. Chromatog., 4 (1960) 169–171

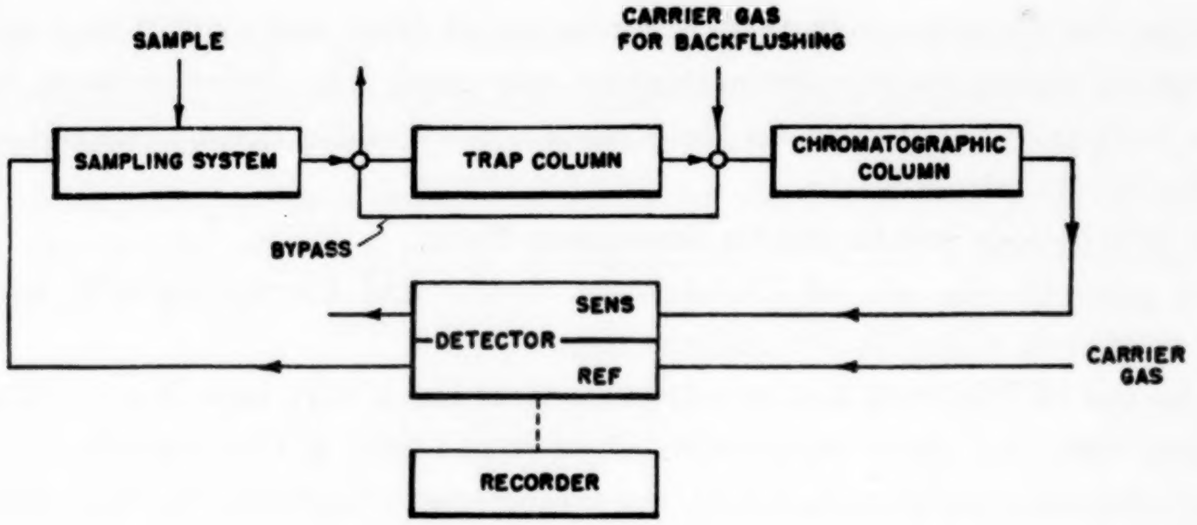

Fig. 1.

There are many limitations if the principles of this method are applied to the more general problem of the analysis of volatile components dissolved in a non-volatile medium. The main problem is that the temperature of the instrument must be kept low, and, therefore, the undesirable condensation (or insufficient evaporation) of the solvent into the injection block or the tubes connecting it with the column will make the analysis difficult for two reasons: (1) a part of the volatiles will remain dissolved in the condensed solvent, and (2) a frequent cleaning of the equipment will be necessary.

Of course, it is possible to heat this part of the instrument using separately installed heating wires, but if commercial gas chromatographs are used, this requires modification on the instrument. It should be further emphasized that, because the necessary sample volume is in most cases unusually large (1 ml: at least 100 times larger than the average liquid sample used at gas chromatographic analyses), the rapid and complete evaporation of this large sample volume is still a problem even if the injection block is heated sufficiently.

A further problem is the backflushing of the trap column which also requires special modification of most commercial gas chromatographs.

Recently, BRENNER AND ETTRE[4] described a device for the analysis of trace impurities in gases by condensing the components of interest in a small column connected to the outside gas sampling valve of a commercial gas chromatograph. A simple modification of this device permits its use for the general problem of the analysis of volatile components dissolved in a non-volatile medium.

The new device consists of a special trap column with a rubber septum on its top (Fig. 2). The column is filled with adsorbents similar to those described in previous papers (activated charcoal, silica gel, firebrick, etc.) or with column materials which show a much longer retention time for the solvent than for the dissolved substances to be analyzed. The large liquid sample is injected using a hypodermic syringe with a longer needle through the rubber septum directly onto the top of the column packing material (valve in position I). In this way, the sample is carried with the carrier gas flow through the trap column; the non-volatile solvent is adsorbed on the packing material, but the volatile components are purged without loss into the chromatographic

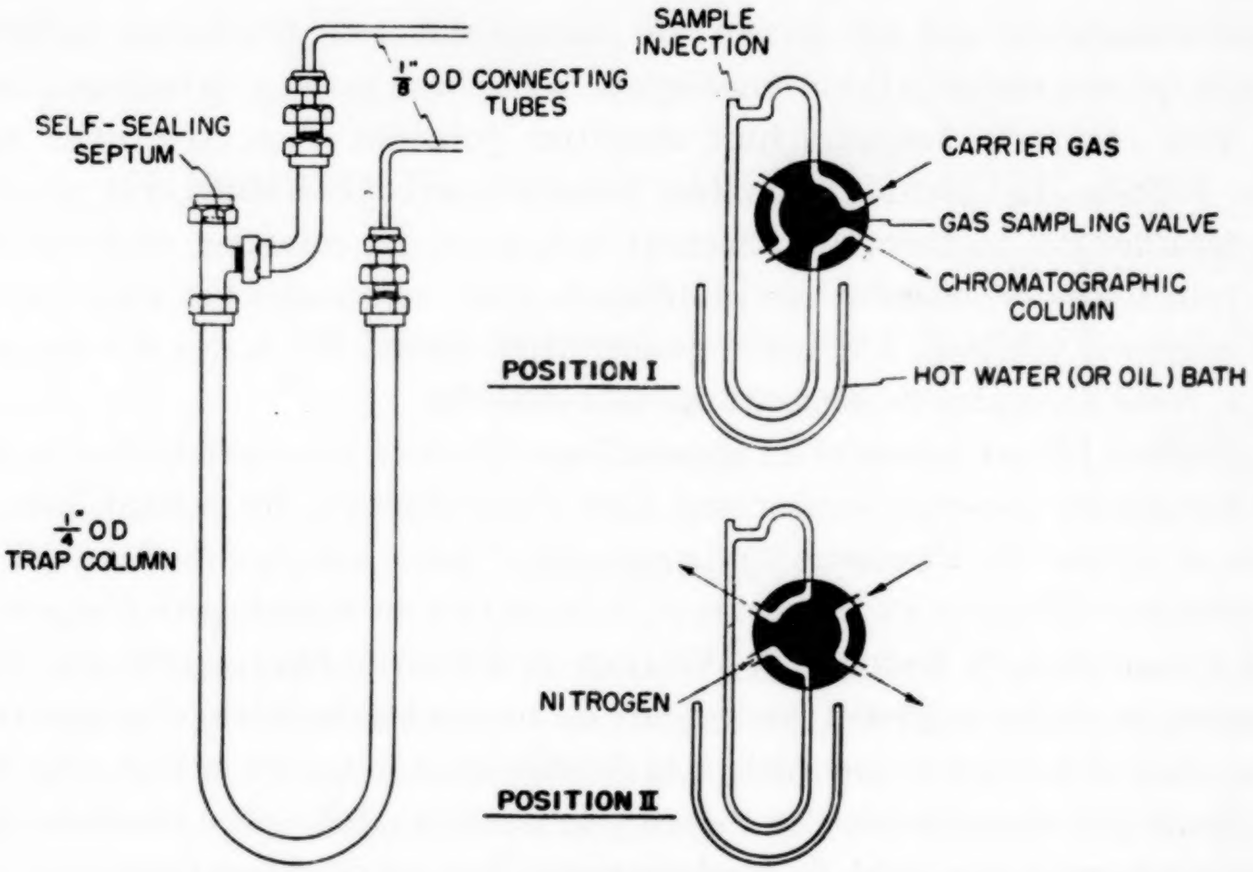
SAMPLE INJECTION
1" / 8 O D CONNECTING TUBES
SELF-SEALING SEPTUM
CARRIER GAS
GAS SAMPLING VALVE
CHROMATOGRAPHIC COLUMN
POSITION I
HOT WATER (OR OIL) BATH
1" / 4 O D TRAP COLUMN
NITROGEN
POSITION II

Fig. 2

column and analyzed there. The trap column may be conveniently heated with a water or oil bath to elevated temperatures if necessary.

After finishing the analysis, the valve is to be turned to position II, and the trap column backflushed using nitrogen or air.

The device has been successfully employed for the analysis of dissolved gases or light organic solvents in water.

The Perkin-Elmer Corporation, Norwalk, Conn. (U.S.A.) EDWARD W. CIEPLINSKI
LESLIE S. ETTRE

[1] P. G. ELSEY, *Anal. Chem.*, 31 (1959) 869.
[2] J. P. PAGLIS, *2nd Biannual International Gas Chromatography Symposium*, Michigan State Univ., June 1959; *ISA Proc.*, 2 (1959) 156.
[3] J. A. PETROCELLI AND D. H. LICHTENFELS, *Anal. Chem.*, 31 (1959) 2017.
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Received April 25th, 1960

BOOK REVIEW

Gas Chromatography (Chromatographie en phase gazeuse), par A. I. M. KEULEMANS, 2e édition, C. G. VERVER, Éditeur, Reinhold Publishing Corporation, New-York, 1959, xxi + 234 pages, 99 figures.

Peu d'années se sont écoulées entre les deux éditions de ce livre, dont l'intérêt, pour ceux qui veulent prendre contact avec cette nouvelle technique, se trouve ainsi démontré. La récente édition marque un progrès sur l'ancienne, en ce sens que l'auteur

a pris soin d'élaguer ce qui est devenu très commercial et de développer certaines des nouveautés qui impriment à la chromatographie en phase gazeuse sa tendance actuelle.

Le livre comporte toujours huit chapitres précédés d'une très utile liste des symboles utilisés. Le premier chapitre, consacré aux généralités, est suivi d'une analyse détaillée des facteurs qui affectent l'efficacité des colonnes, et d'une description des principales représentations graphiques grâce auxquelles l'analyse qualitative d'un mélange est possible. L'analyse quantitative donne lieu à des développements suffisants. Neuf exemples de séparations sont discutés.

Le chapitre III est consacré à l'appareillage. On note avec satisfaction la description de techniques récentes concernant tant l'introduction des échantillons que la détection au moyen de détecteurs "hypersensibles" par exemple à décharge électrique à basse pression (HARLEY ET PRETORIUS), à ionisation en atmosphère d'argon (LOVELOCK) et à ionisation de flamme (MCWILLIAM ET DEWAR). On regrette que les délais d'impression de ce livre privent les lecteurs de toutes les dernières découvertes en ce domaine, mais il faut bien consentir à se limiter dans le temps si l'on veut réaliser.

Le chapitre suivant traite des théories de la chromatographie en phase gazeuse, et n'a pratiquement pas subi de modifications. Il en est de même pour ceux qui sont consacrés à la phase mobile, et à l'ensemble support solide–phase liquide stationnaire.

Le chapitre VII, réservé aux applications les plus récentes, consacre plusieurs pages nouvelles aux améliorations apportées par SCOTT aux colonnes classiques et à l'introduction par GOLAY des colonnes capillaires.

Le dernier chapitre passe en revue les techniques et les applications de la chromatographie d'adsorption en phase gazeuse, et rend aux travaux de CRAMER et de son école la place qui leur est due.

On trouve encore deux appendices dont le premier concerne le choix pratique des phases stationnaires. Une liste, avec températures limites d'emploi, peut servir de guide. Volontairement réduite par l'auteur, elle l'a peut être été dans de trops grandes proportions: on y cherche en vain le β,β'-oxydipropionitrile, si employé. Le second appendice donne une technique d'élimination des impuretés gazeuses du gaz carbonique commercial servant de gaz vecteur en chromatographie d'adsorption. Enfin, le livre se termine par une table des auteurs cités et une table des matières bien conçue.

De lecture facile, composé au moyen de caractères agréables à l'oeil, largement illustré, cet ouvrage connaîtra sans nul doute le même succès que celui qui l'a précédé. Il n'est pas, à l'heure actuelle, de meilleur guide dans un domaine où l'évolution est rapide et où il est nécessaire, de temps en temps, de faire le point des connaissances fondamentales.

P. CHOVIN

REVIEW

CHROMATOGRAPHY OF LIPIDS ON SILICIC ACID

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(Received March 21st, 1960)

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INTRODUCTION

Twenty years have passed since KAUFMANN[1] and TRAPPE[2] first reported chromatography of lipids on silicic acid. For ten years relatively little further work was reported[3-18], but after 1950 the number of references in the literature to this technique rose roughly exponentially, until in 1959 (when there were well over 100) the rise clearly tapered off. It seems appropriate now to summarize what has been learnt about silicic acid chromatography of tissue lipids and similar compounds that are of interest.

Development of chromatographic methods has made most problems in lipid chemistry and biochemistry more tractable than they were previously. The problem of resolving lipid extracts from natural sources on a preparative scale into separate, intact, molecular species has been investigated most successfully by column chromatography on silicic acid. Whether it could be completely solved by using silicic acid or any other adsorbent is a matter for speculation, or rather, for future investigation. The work of TRAPPE[3], BORGSTRÖM[19-21], FILLERUP AND MEAD[22, 23], and LEA, RHODES AND STOLL[24] established that a silicic acid column can separate a natural lipid mixture into two classes, "neutral lipids" and phospholipids, and can resolve these into more classes (*e.g.* triglycerides, sterols, etc.). This ability to differentiate between lipid classes, largely uninfluenced by olefinic unsaturation, accounts chiefly for the present-day popularity of silicic acid as an adsorbent. Moreover, since good silicic acid adsorbents are unreactive at room temperatures, lipids can be recovered after chromatography in yields of 100%, within limits of error in measurement. Labile compounds, such as hydroperoxides[25], allylic tertiary alcohols[26-28], and plasmalogens are safe.

The bibliography in this review is restricted by avoiding reference to papers that mention merely the use of methods described elsewhere, and in the section on "The Adsorbent" it is intentionally less than comprehensive.

THE ADSORBENT

Silica, *silica gel*, and *silicic acid* are terms used synonymously in the literature[29]. They refer to powdered solids of the general formula $SiO_2 \cdot xH_2O$ which consist of porous, three-dimensional siloxane (tetrahedral Si·O·Si) structures with surface silanol (Si·OH) groups. There is usually a mono- or multi-molecular layer of adsorbed water, but such "free" water can be removed reversibly by heating at about 100°. At temperatures above 170° condensation of silanol groups occurs, liberating "fixed" water, and this process of thermal degradation continues with rising temperature until, at 1100°, only SiO_2 remains. Thermal degradation is rather irreversible because it destroys pore structure, and it permanently impairs adsorbent properties[30-42].

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The behaviour of a sample of silicic acid as a chromatographic adsorbent depends on its content of silanol groups, free water, and chemical impurities, and on topography (including pore size and shape) and particle size.

The fundamental adsorbent property of silicic acid is attributed to the silanol groups, which in absence of adsorptive (*e.g.* free water) are free of hydrogen bonding[43, 44]. Affinity for an adsorptive is governed *primarily* by the strength of hydrogen bonding possible with it[34, 35, 44-49]. A sample of silicic acid is fully activated as an adsorbent if devoid of free water but not thermally degraded. It is progressively deactivated by water, remarkably large quantities of which can be added before the powder becomes visibly wet. Occasionally, chromatographic resolution is improved by adding free water to the adsorbent and using a less polar eluent[50, 51]. Using non-hydroxylic eluents on silicic acids containing 0 to *ca.* 17% free water the process governing chromatography is adsorption at a solid surface, whereas on silicic acids containing larger amounts of free water it is liquid–liquid partition. (This review is concerned primarily with adsorption chromatography.)

For use as an adsorbent silicic acid should be as pure as possible, since traces of impurity may appear in eluted fractions or may catalyze formation of artefacts. WALLING[52] has shown that carefully prepared silicic acids have neutral surfaces, but others have acidic surfaces due to sulphuric acid remaining after preparation.

Silicic acid prepared from alkali silicates has a porous structure which is vitally important in chromatography. Studies on hydrocarbons illustrate that considerations of topography far outweigh those of gross surface area in determining capacity and selectivity for an adsorptive[53, 54]. Fine grinding of the adsorbent improves chromatographic resolution[55], but since it is tedious to prepare extremely fine powders, and since such powders form columns that are impervious to eluents, a compromise has to be made.

The above considerations show why different preparations of silicic acid may have widely different adsorbent properties, a fact that has troubled many workers. For chromatography of tissue lipids two or three American commercial preparations labelled *silicic acid* have proved far superior to others, particularly to others labelled *silica gel*. (This is the apparently fortuitous reason why most workers on lipids prefer the term *silicic acid chromatography* to the term *silica gel chromatography*. Objections can be raised to the use of either term.)

LOVERN and co-workers[56-59] have reported a remarkable contrast between the behaviour of phospholipids on columns of *silicic acid* (Mallinckrodt) and *silica gel* (B.D.H.). Whereas phospholipids are eluted after neutral lipids from *silicic acid*, they are eluted first from *silica gel, being unadsorbed from ligroin* solution.* RILEY AND NUNN[60] have reported similarly, except that urea as well as phospholipids occurred in their ligroin eluate from *silica gel* and some phospholipids were found eluted after neutral lipids (see also LOVERN *et al.*[57]). BLOCH, LEDERER, and co-workers[61–65] have reported that "cord factor" (trehalose 6,6'-dimycolate) and related polyhydroxy lipids are strongly adsorbed from ligroin solution on *silicic acid* (Mallinckrodt) but are largely unadsorbed on *silica gel* (Davison). The small amounts of these lipids that are adsorbed on *silica gel* can be eluted by solvent mixtures that would elute them from *silicic acid.* These observations can all be explained as follows. Phospholipids and other lipids containing polar groups exist in ligroin solution in micelles with hydrocarbon surfaces, and the micelles are broken up readily by *silicic acid* but with difficulty by *silica gel.* Intact micelles are eluted as if they were hydrocarbon macromolecules. Non-lipids, such as urea, may be included in the micelles (the well-known "solublizing" effect).

Comparing adsorption on samples of *silica gel* (Davison) and *silicic acid* (Mallinckrodt), CINES AND RUEHLEN[66] obtained Type I isotherms for the former and Type II for the latter: whereas *silica gel* showed only monolayer adsorption of 2,4-dimethylpentane and benzene vapours, *silicic acid* showed adsorption beyond the monolayer, although selectivity for adsorptive resided mainly in the monolayer. The relatively high silanol content of the *silicic acid* (fixed water = 4.75%[48]), sulphate impurity in the *silica gel,* topography (*e.g.* pore size distribution[66]), or a combination of these factors may account for the striking difference. In any case, it seems that whatever causes *silicic acid* to adsorb more than a monolayer of hydrocarbon may also allow it to break up micelles of polar lipids.

Lipids that do not contain highly polar groups (neutral lipids) are eluted in the same order—and by approximately the same solvent mixtures—from *silica gels* as from *silicic acids.*

For some purposes commercial silicic acid preparations may be used as received, but to obtain best and most reproducible results they should be pre-treated. HIRSCH AND AHRENS[67] have published a very valuable paper in which is described a method of pre-treatment that yields adsorbent of standard, high quality. Its particle diameter is small ($\lesssim 40\ \mu$), it does not unduly restrict the flow rate of eluent, and it is substantially devoid of free water and chemical impurities. HIRSCH AND AHRENS recommend that columns of this adsorbent should be washed with acetone–ether (1:1**) and ether before use. The acetone–ether removes free water, and the ether removes acetone. Removal of free water by means of dehydrating washes has often been advocated, and non-hydroxylic solvents, such as pure acetone or dioxan, apparently can be displaced by the first eluent (*e.g.* ether) before chromatography begins. Methanol and ethanol have been used in dehydrating washes, often on the mistaken assumption that they, too, can be similarly displaced. But at least a monolayer of alcohol is tenaciously retained, and this affects the adsorption of lipids[48, 68, 69]. It seems that if methanol washes are employed some phospholipids are prematurely eluted by ether[70-72].

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* For purposes of this review, ligroin = light petroleum = petroleum ether = benzine.

** Eluent compositions are given by volume. In the particular case of chloroform–methanol mixtures, volumes of the components are roughly additive: thus, for example, *25% methanol in chloroform* and *chloroform–methanol (3:1)* describe essentially the same eluent.

At 100° silicic acid is quite reactive, since siloxane oxygen, as well as silanol oxygen, exchanges with the oxygen of $H_2{}^{18}O$.[33] For practical purposes heating at 100-120° removes all volatile compounds, but in certain circumstances this might not yield a fully active adsorbent since some silanol groups condense with alcohols under mild conditions[73-75]. Deliberate modification of silicic acid, by alkylation, arylation, acylation, or siliconization, might actually provide useful new adsorbents[41, 74, 76, 77]. Another kind of modification might be useful for specific separations or for studies of configuration: a silicic acid precipitated in presence of a chosen compound may possess specificity for that compound and for others with similar structural features[35, 78, 79].

Admixture of filter aids with silicic acid adsorbents, although frequently mentioned in the literature, should be avoided. As HIRSCH AND AHRENS[67] have pointed out, they introduce an unwanted variable in adsorbent quality, and adequate elution rates are possible without them.

Although pure silicic acid has a neutral surface[52], it has greater affinity for amino groups than is attributable to hydrogen bonding alone[49, 80]. It also has limited cation exchange properties which are troublesome in chromatography of acidic phospholipids (see below)[80-85].

ELUENTS

Methanol > ethanol > 1-propanol > acetone > methyl acetate > ethyl acetate > ether > dichloromethane > benzene > toluene > 1,1-dichloroethane > 1,1,2,2-tetrachloroethane > chloroform > trichloroethylene > carbon tetrachloride > cyclohexane > ligroin.

Above is an expanded and slightly modified version of TRAPPE's "eluotropic series", listing common solvents in order of their eluting power[2, 86, 87]. The order varies slightly according to the adsorbent sample and the adsorptive studied. The solvents most commonly used are methanol, ether, benzene, chloroform, and ligroin, though there seems no reason why others, including dioxan and 2-methoxyethanol, should not prove useful.

The literature provides some information on the relative merits of different eluents for particular separations. But it may sometimes be worthwhile to compare adsorption values of purified lipids from several solvent mixtures before selecting a mixture as an eluent[2, 13, 67]. Alternatively, eluents can be compared by using them in chromatography on silicic acid impregnated glass fibre paper (see Table I)[88-94]. This is a better model of column chromatography than chromatography on impregnated filter paper or chromatoplaques, which consist of polysaccharides besides silicic acid. It is not a perfect model, however, because frontal analysis of the eluent is possible. Table I illustrates that the order of elution of lipids may vary with the eluent used: for example, in isoöctane–ether vitamin A palmitate moves more slowly than hexadecanol, but in cyclohexane–methanol it moves faster.

TABLE I

R_F VALUES OF LIPIDS ON SILICIC ACID-IMPREGNATED GLASS FIBRE PAPER

Reference	*92*	*92*	*89*	*92*	*90*	*91*
Solvent	*A*	*B*	*C*	*D*	*E*	*F*
Squalene	0.92	0.94		0.90	1	
Hexadecyl palmitate	0.61	0.93		0.91	1	
Vitamin A palmitate	0	0.46		0.87	1	
Cholesteryl palmitate	0.63	0.90	0.93	0.93	1	
Cholesteryl acetate			0.96		1	
α-Tocopheryl acetate	0.32	0.83		0.91	1	
α-Tocopherol	0.42	0.79		0.91	1	
Tripalmitin			0.93		1	
Triglycerides (unsaturated)	0	0.65	0.89	0.89	1	
Hexadecanol	0.16	0.65		0.64	1	
Vitamin D_3	0.07	0.55		0.74	1	
Vitamin A alcohol	0	0		0	1	
Stearic acid, linoleic acid					1	0.95
Cholesterol	0.08	0.44	0.67	0.69	1	
7-Dehydrocholesterol	0.04	{0 0.38		0.48	1	
1,3-Dipalmitin			0.53		1	
1,3-Diolein			0.48		1	
1-Monopalmitin	0		0.11		1	
1-Monoölein	0		0.11		1	
Phosphatidylethanolamines	0	0	0		0.94	0.85
Lecithins (from egg)	0	0	0	0		0.66
Lecithin (dipalmitoleyl-)	0	0	0	0	0.50	
Lecithin (distearyl-)	0	0	0	0	0.46	
Lecithin (dimyristyl-)	0	0	0	0		0.65
Sphingomyelins	0	0	0	0	0.40	0.50
Lysolecithins	0	0	0	0		0.31
Lysolecithin (2-palmitoleyl-)	0	0	0	0	0.23	
Lysolecithin (2-stearyl)	0	0	0	0	0.18	

R_F values differ slightly on different batches of impregnated paper. Mixtures of compounds with close R_F values (*e.g.* the lysolecithins) have not been resolved.

Solvents. A: cyclohexane. B: 2% ether in isoöctane. C: 5% ether in isoöctane. D: 1% methanol in cyclohexane. E: 50% methanol in ether. F: phenol (200 g) + ether (400 ml) + acetone (250 ml) + water (50 ml).

For chromatography of natural lipid mixtures some form of gradient elution is usually employed, namely, continuous or discontinuous, convex, linear, or concave gradient elution (Fig. 1). Most workers have hitherto used discontinuous concentration gradients, in spite of their tendency to give factitious peaks in elution curves. Columns require less attention when continuous concentration gradients are used. Moreover, the symmetry and resolution of peaks is improved *if a suitable gradient is chosen.* A continuous convex gradient of methanol in chloroform resolves cerebrosides adequately[95, 96] but phospholipids very poorly[97]; the latter are resolved well by a continuous concave gradient[98]. For resolution of as many components of a natural

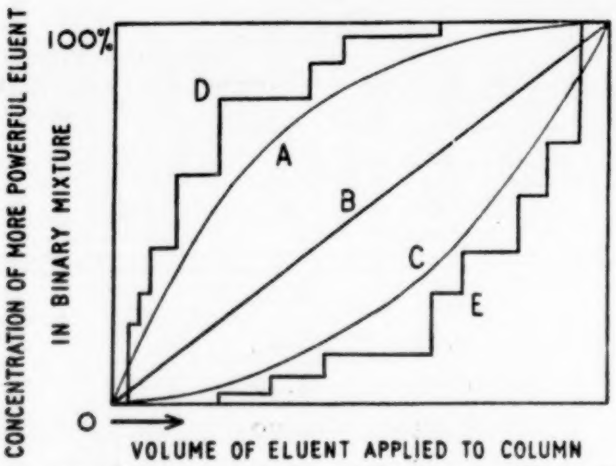


Fig. 1. Concentration gradients: continuous convex (A), linear (B), and concave (C); discontinuous convex (D) and concave (E).

lipid mixture as possible two or more successive gradient elutions are necessary, *e.g.* ligroin → chloroform → methanol or ligroin → ether → ethanol → methanol.

Eluents should be of analytical reagent grade or purer. Chloroform when purchased contains, as a preservative, 0.75–2% ethanol which gradually saturates a silicic acid column (the R_F value of ethanol in chloroform is low[99]). The effect on elution behaviour is remarkably slight, but sometimes it is worthwhile to remove the preservative just before use.

EXPERIMENTAL CONSIDERATIONS

For general information on techniques see LEDERER AND LEDERER[29], KORN[100], NEHER[101], and HIRSCH AND AHRENS[67].

Lipids must be chromatographed with considerable care, to avoid degradation and contamination as far as possible. Natural lipid mixtures are particularly demanding, since they require long elution periods (*e.g.* 200 hours) and large volumes of eluents (*e.g.* 150 ml per gram of adsorbent). HIRSCH AND AHRENS[67] suggest that control of column temperature is important. Adsorbent should never be re-used after washing, because contamination could result, and previously used eluents such as alcohols cannot be entirely washed away.

The susceptibility of lipids, particularly tissue phospholipids, to autoxidation makes it desirable to chromatograph them anaerobically. Therefore some workers have used nitrogen or carbon dioxide atmospheres and deoxygenated adsorbents and eluents[16, 56, 57, 70, 102–110]. The adsorbents were deoxygenated thermally or by washing with hydroquinone[109] or linoleic acid[56, 57] solutions.

Overloading of columns, which vitiated some early work, must be avoided. The load of a natural lipid mixture should not exceed 50 mg per gram of adsorbent, but frequently one tenth of this load is preferred. The load of a lipid preparation that has already been fractionated should be lower.

When a particular type of lipid has to be prepared it is worthwhile to choose a source in which that type is abundant, since adsorbent, eluents, and time can thus be saved. The purity of the product can be improved by re-chromatography. For example, to prepare a lecithin sample an extract from hen's eggs, freed of non-lipid contaminants by an appropriate method, can be run on to a column in 15% methanol in chloroform; after prolonged elution with this mixture, 20% methanol will elute the lecithin peak, and this can be re-chromatographed.

It is better to support the adsorbent on a sintered glass plate and/or a glass wool plug than on a filter paper or a cotton wool plug, because cellulose adsorbs polar lipids from some eluents[97]. Using suitable adsorbent samples (see above) in columns with moderate height: diameter ratios (*e.g.* 5:1), the rate of flow of most eluents is satisfactory. If pressure or suction is ever applied to improve the rate of flow it should be moderate, otherwise the adsorbent packs down tighter and so defeats the purpose[97]. If the rate dwindles after prolonged elution it can sometimes be speeded by cautiously stirring the adsorbent to a depth of a few millimetres[111].

Automatic fraction collectors activated by direct volume measurement are preferable to those activated by weight, drop counting, or timing, because the density, surface tension, and rate of delivery of eluate are very considerable variables. Eluate measuring devices and delivery tubes should be designed to minimize evaporation, which causes lipid to accumulate on them.

The course of elution can be followed by one or more of numerous methods. The best single method, although laborious, is weighing the involatile portion of each fraction in a tared vessel. "Piloting" by paper chromatography, by using chromatoplaques, or by paper electrophoresis, is very widely used, as are colorimetric analyses of cholesterol, esters, aldehydes, amino compounds, and phosphorus. Infrared and ultraviolet spectrophotometry, radioactivity measurements, and hydrolytic methods are also used. It is sometimes possible actually to watch bands being eluted, as when chloroform is the eluent and the column is almost transparent.

It might be expected that the free amino acids, sugars, etc. that contaminate lipid extracts from natural sources would be retained more firmly by silicic acid than any lipid. Unfortunately this is not so[97, 111-114]. Thymine, for example, is eluted before phosphatidylethanolamines[115], and urea and glucose after, followed by numerous amino acids. In a model experiment in which solid non-lipids were placed on the top of a column in absence of lipids, WREN AND MITCHELL[111] found that 15% methanol in chloroform eluted 2-aminoöctanoic and 2-pyrrolidone-5-carboxylic acids, then glucose, cystine, leucine, and proline. Higher concentrations of methanol eluted more amino acids sequentially, and only cysteine, histidine, and lysine hydrochlorides were retained from pure methanol. (There was necessarily much tailing due to insolubility of these compounds in the eluents.) To avoid misleading results, non-lipid contaminants should be removed from lipid extracts before chromatography. For this purpose, selective precipitation is not effective since polar lipids form micelles around non-lipids in non-polar media. Cellulose chromatography is effective but it removes relatively polar lipids such as inositides. Liquid–liquid partition methods are also effective and are widely favoured, although they do involve losses of the most polar lipids. The FOLCH (1957) method, about which most is known[116-123], employs the system chloroform–methanol–water, and the method of BLANKENHORN AND AHRENS[124, 125] employs heptane–alcohol–water mixtures. Both methods are combined with reproducible extraction procedures, and in both partition can easily be repeated if necessary.

Traces of adsorbent are washed during chromatography from even the most carefully prepared columns[67, 72, 95, 111, 114, 126–128]. It is not clear whether the reason for this is mechanical, or whether it is combining or dissolving effects of lipids[129] or eluents. Neither is it clear whether or not some silicon found in eluates is tissue silicon originating with the lipids[35, 129]. If eluates are evaporated and the residual lipids redissolved, silica can be removed by centrifugation or filtration[95, 126, 127]. Silica may interfere in phosphorus analysis[130], but it can conveniently be discarded after perchloric acid digestion[131].

The possibility of contamination by silicon and other elements should be borne in mind when fractions from silicic acid columns are used in biochemical systems. Lecithin fractions eluted from silicic acid are acidic enough to resist lecithinase A[97, 132].

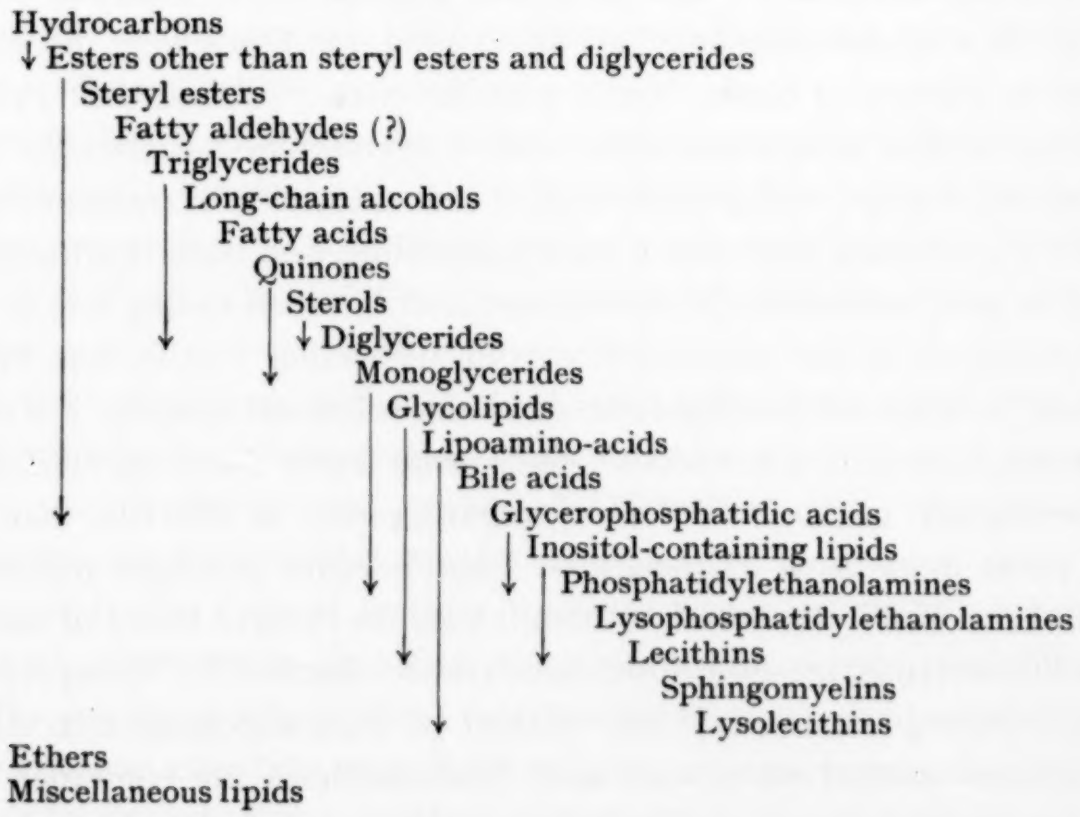
Although the emphasis of this review is on methods for preparative resolution of lipid mixtures, the use of silicic acid in biochemical microanalysis deserves mention. Micro columns (containing 0.5–1.0 g adsorbent) and impregnated papers are used widely. Phospholipids can be separated from neutral lipids by simply shaking a chloroform solution with silicic acid, which adsorbs phospholipids and can be separated by centrifugation or filtration[133–135].

ORDER OF ELUTION

The list in Table II is presented in the order of elution of the most readily eluted member of each lipid class. The arrows give some idea of the behaviour of less readily eluted members.

TABLE II

ORDER OF ELUTION OF LIPIDS FROM SILICIC ACID



Hydrocarbons
↓ Esters other than steryl esters and diglycerides
Steryl esters
Fatty aldehydes (?)
Triglycerides
Long-chain alcohols
Fatty acids
Quinones
Sterols
↓ Diglycerides
Monoglycerides
Glycolipids
Lipoamino-acids
Bile acids
Glycerophosphatidic acids
Inositol-containing lipids
Phosphatidylethanolamines
Lysophosphatidylethanolamines
Lecithins
Sphingomyelins
Lysolecithins
Ethers
Miscellaneous lipids

In general, higher homologues are eluted more readily than lower homologues. Elution is retarded slightly by olefinic unsaturation, more by electronegative substituents, and most by substituents containing hydrogen atoms capable of hydrogen bonding. Further generalization is difficult, because elution behaviour is influenced by specific spatial and electronic interactions between adsorbent, eluent and adsorptive.

Each lipid class will be discussed in order.

Hydrocarbons

The saturated hydrocarbons of tissues are found in the first fraction from silicic acid, and non-polar solvents (pentane, hexane, isoöctane, ligroin, carbon disulphide) elute them before other lipids[56, 57, 67, 104, 111, 128, 136–141].

It can be assumed that hydrocarbons are eluted sequentially according to the inverse order of adsorption, acyclic alkanes > cycloalkanes > straight chain monoenes > branched chain and cyclic monoenes > dienes > benzenoid hydrocarbons and monoynes > polyenes[32, 56, 138, 139, 142–149], and under suitable conditions hydrocarbon mixtures can be fairly well resolved. SMIT[143, 144] eluted cetane before cetene with pentane, then dodecylbenzene with carbon tetrachloride. Using hexane as developing solvent on silicic acid–starch chromatostrips, KIRCHNER AND MILLER[146] found widely differing mobilities among a selection of terpenoid and other hydrocarbons. CLEMENTS[141] was able to separate *p*-menthane, α-pinene, β-pinene, and *d*-limonene (eluted in this order) on a silicic acid column at —78°. A silica gel column 16 metres high partly resolved mixtures even such as 2,2,4-trimethylpentane–*n*-octane, *n*-dodecane–*n*-heptane, and cyclohexane–cyclopentane[142].

The polyenes, carotenes[67, 139, 150, 151] and squalene[67, 92, 138, 152], are not eluted by pure hydrocarbon solvents. HIRSCH AND AHRENS[67] found that β-carotene and squalene were eluted by 1% ether in ligroin in a fraction containing wax esters, but NICOLAIDES AND FOSTER[138] eluted squalene well before and SORRELS AND REISER[92] well after wax esters. Results of chromatography on silicic acid-impregnated glass fibre filter paper (Table I) seem to confirm that the order of elution of these compounds is sensitive to small differences in adsorbents and eluents.

A method for quantitative determination of carotene in green leaves makes use of the fact that carotene is less strongly adsorbed than the other pigments present[153].

Esters other than steryl esters and glycerides

The naturally occurring wax esters[56, 57, 67, 92, 111, 137, 138, 141, 152] (*e.g.* hexadecyl palmitate[92, 152]) can be eluted between the hydrocarbons and steryl esters if column resolution is good. Most workers have found a slightly polar eluent necessary (*e.g.* 1% ether in ligroin[67, 92, 152]); SPENGLER AND HAUF[137] found that octadecyl stearate (also 4-hydroxystearolactone) was retained from ligroin or carbon disulphide, but eluted by carbon tetrachloride.

In 1949, RIEMENSCHNEIDER *et al.*[16] described the isolation of pure linoleic and linolenic acids by means of an elegant chromatographic experiment in which the methyl esters were fractionated on silicic acid–Hyflo Supercel (4:1, w/w) under strictly anaerobic conditions. Further work has confirmed that fatty acid methyl[18, 67, 102, 103, 105–108, 154, 155] (and ethyl[107, 137, 156]) esters are generally eluted in order of increasing unsaturation, first by ligroin and then by low concentrations of ether[67, 102, 103, 105–108, 155] or benzene[154] in ligroin. Thus, a methyl isolinoleate has been separated from saturated and monoene esters[18], methyl arachidonate prepared from beef glands[102] and pork liver[108], and a methyl docosahexaenoate prepared from hog brain[106]. Higher homologues tend to be eluted first: for example, methyl docosapentaenoate precedes methyl eicosapentaenoate[103], and the adsorption isotherm of methyl stearate lies below that of methyl myristate[154]. Though methyl *cis*- and *trans*-oleates have not been separated quantitatively, it is interesting that the *trans*-oleate (elaidate) tends to be eluted first[105, 157]. The behaviour of methyl isanate[158] suggests that acetylenic bonds influence the order of elution only slightly. Hydroxyl substituents, on the other hand, greatly retard elution. BERGSTRÖM AND PÄÄBO[159] have found that methyl palmitate and other unsubstituted esters are eluted by pure dichloromethane, whereas methyl *threo*-9,10-dihydroxystearate and other dihydroxy esters require 0.75% methanol, and tetrahydroxy esters require 2–3% methanol in dichloromethane (which is sufficiently polar to elute some phospholipids). Bromo substituents slightly retard elution, and methyl *threo-threo*-9,10,12,13-tetrabromostearate tends to be eluted after methyl stearate[160]. R_F values recorded[94] for methyl polybromostearates on silicic acid-impregnated glass fibre paper in isoöctane are: hexa-, 0.23; tetra-, 0.47; di-, 0.62; *cf.* methyl oleate, 0.78.

p-Phenylphenacyl[161] and *p*-phenylazophenacyl[162] esters of saturated fatty acids are eluted (as fluorescent and orange bands, respectively) by ligroin–benzene mixtures in order of decreasing molecular weight. Useful separations can be obtained, *e.g.* stearate from myristate[162].

Phytyl acetate and azobenzoate[28], α-tocopheryl acetate[92], vitamin A acetate[67], and vitamin A palmitate[92, 152] are other esters of this group that have been chromatographed on silicic acid. Vitamin A acetate is somewhat difficultly eluted, and appears in the monoglyceride fraction eluted by pure ether. MARINETTI *et al.*[163] have prepared from heart muscle lipids a new keto ester, which was eluted from silicic acid by 3% ether in ligroin.

Steryl esters

Cholesteryl esters can be obtained in a well resolved fraction before the triglyceride fraction[3, 20, 22, 23, 56, 60, 67, 72, 92, 98, 104, 111, 112, 120, 127, 128, 138, 139, 152, 164–167], the preferred eluent being 1% ether in ligroin[22, 23, 60, 67, 92, 104, 139, 152, 167]. The similarity of the R_F values of cholesteryl acetate and palmitate on silicic acid-impregnated glass fibre paper (Table I) illustrates the difficulty of column separation of steryl esters of saturated fatty acids, which has only once been reported (cholesteryl palmitate was eluted before the myristate[168]). Steryl esters of unsaturated fatty acids are more easily separated[56, 168], and KLEIN AND JANSSEN[168] have eluted cholesteryl palmitate, oleate, linoleate, and arachidonate sequentially, with 15% benzene in hexane. Apparently iodine-labelling of cholesteryl esters does not markedly affect their elution behaviour[169, 170].

The quantitative separation of cholesteryl esters from free cholesterol that can be effected on silicic acid, together with colorimetric analysis, facilitates micro-scale study of relative amounts of these compounds in tissues (see *e.g.* refs.[164-166, 168, 171-173]). However, autoxidation in samples must be strictly avoided, since autoxidation products are eluted late[174].

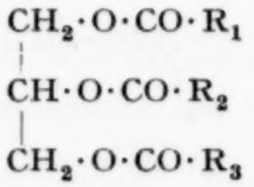
Chromatographic resolution of mixtures of closely related sterols can be facilitated by prior esterification. Thus, STOKES *et al.*[175-177] separated the *p*-iodobenzoates of cholestanol, cholesterol, and Δ^7-cholestenol (decreasing order of mobility). STOKES[178] showed mobilities, dihydrolanosteryl > dihydroagnosteryl > lanosteryl > agnosteryl, for benzoates and *p*-iodobenzoates. Δ^7-Cholestenol has been isolated from skin[179] and faeces[180] as its azoate, which forms a band above that formed by cholesteryl azoate. From 3,5-dinitrobenzoylated urinary steroids KELLIE AND WADE[181] obtained the diester of pregnane-3α,20α-diol, which was eluted after the ester of cholesterol.

Fatty aldehydes

Aldehydes liberated from plasmalogens are conveniently separated from phospholipids by elution with chloroform[182, 183]. It is not known whether they are eluted as monomers or as polymers, or indeed what is their precise position in the order of elution of lipids. However, considerations of chain length and unsaturation, discussed above for esters, apply in the same way to aldehydes[184].

Triglycerides

Since the early work of TRAPPE[3] and KAUFMANN and co-workers[6-9] reports of silicic acid chromatography of triglycerides have been numerous[19-23, 56, 57, 60, 67, 72, 92, 98, 104, 111, 112, 120, 127, 128, 138, 139, 152, 156, 167, 169, 185-191]. Higher triglycerides have most often been eluted with 3–5% ether in ligroin[22, 23, 60, 67, 92, 104, 128, 139, 152, 167] or with benzene[3, 6-9, 21, 138, 185, 188, 189]. Lower triglycerides are eluted after higher[7-9, 67]. Experimenting with a selection of lipids, HIRSCH AND AHRENS[67] obtained tristearin, trilinolein, tricaproin (I, $R_1 = R_2 = R_3 = (CH_2)_4 \cdot CH_3$), and "monoacetin fat" (I, R_1 or $R_2 = CH_3$, R_2 or R_1 and R_3 long) in one fraction, "diacetin fat" (I, $R_1 = R_2$ or $R_3 = CH_3$, R_3 or R_2 long) in the next (with sterols), and tributyrin (I, $R_1 = R_2 = R_3 = (CH_2)_2 \cdot CH_3$) in the next (with dipalmitin and dilaurin); tricaproin formed a well resolved peak after tristearin. By analogy with hydrocarbons and non-glyceride esters (see above) the mobility of triglycerides is generally thought to be reduced by unsaturation[56] (contrast the suggestion of MILLER AND COOPER[120]). On silicic acid-impregnated glass fibre paper the R_F value of unsaturated glycerides is 3–4% lower than that of tripalmitin (Table I).



$$\begin{array}{l} CH_2 \cdot O \cdot CO \cdot R_1 \\ | \\ CH \cdot O \cdot CO \cdot R_2 \\ | \\ CH_2 \cdot O \cdot CO \cdot R_3 \end{array}$$

(I)

Iodine-labelling in triglycerides has no appreciable effect on elution behaviour[169, 187, 191].

Long-chain alcohols

Under suitable conditions saturated, long-chain alcohols can be eluted after triglycerides[92, 152] and before free fatty acids[137, 152]. Eluents that have been used are 2% acetone in ligroin[141], 7% ether in pentane[140], 1% *tert.*-butanol in benzene[192], and chloroform (1-octadecanol is retained from carbon tetrachloride)[137]. Although resolution on their columns was incomplete, Reiser and co-workers[92, 152] have demonstrated the order of elution, triglycerides > hexadecanol > vitamin A alcohol > cholesterol > vitamin D_3 (see also Table I). Kates and Eberhardt[150] have found xanthophylls in chloroform eluates of green leaf lipids. Demole and Lederer[26-28] have eluted acyclic diterpenoids with benzene in the order, phytyl acetate > isophytol (monoene, tertiary alcohol) > geranyl-linaloöl (tetraene, tertiary alcohol) > phytol (monoene, primary alcohol). This order suggests steric hindrance of adsorption of the tertiary alcohols.

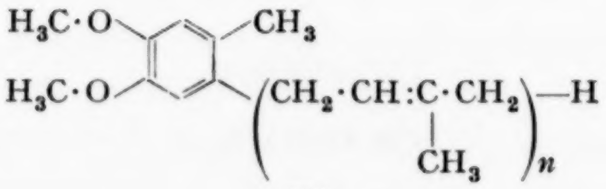
Fatty acids

The question whether free fatty acids of tissues can be separated from triglycerides on silicic acid and, if so, whether they are eluted before cholesterol, has been controversial[3, 6, 19, 20, 22, 23, 67, 104, 128, 139, 152, 167]. But in the carefully conducted experiments of Hirsch and Ahrens[67] free fatty acids were quite cleanly separated in a peak between the triglyceride and cholesterol peaks. Fatty acids in hydrolysates can readily be separated from more polar compounds[193-195].

The usual effects of chain length and unsaturation (see above)[196] allow at least partial resolution of mixtures of stearic, myristic, and lauric acids[1, 10, 11], and stearic and oleic acids[15]. More remarkable (*cf.* methyl esters, above) is the effect of geometrical isomerism: *trans*-oleic (elaidic) acid moves faster than *cis*-oleic, and *trans*-erucic (brassidic) faster than *cis*-erucic[9]. Hydroxy acids are eluted late[61, 62, 193-197].

Quinones

It seems that Coenzymes Q (II) are eluted just after triglycerides and free fatty acids, and that at least some fractionation among them (different values of *n*) is possible[198].



(II)

Eluents that have been used are chloroform–hydrocarbon mixtures[198-200] and 2–5% ether in ligroin[201].

Among the lipids of *Mycobacterium tuberculosis* two naphthoquinone derivatives have been found, one (K_x) eluted in triglyceride fractions and the other (K_y) in a fraction containing monoglycerides[190, 202].

The use of silicic acid chromatography in isolation of plant quinones (*e.g.* see ref.[203]) deserves mention.

Sterols

In chromatography of animal lipids cholesterol is eluted after triglycerides as the next major fraction[3, 22, 57, 60, 67, 72, 92, 98, 111, 112, 128, 139, 151, 152, 167, 204], which is separate even when an unnecessarily polar eluent such as chloroform is used[98, 111, 112]. A preferable eluent is 10% ether in ligroin[22, 60, 67, 92, 139, 140, 151, 152, 167, 204], several column volumes of which are needed because the cholesterol peak "tails"[67]. Coprostanol (saturated) can be eluted before cholesterol[180] and β-sitosterol[67] (monoenes); 7-dehydrocholesterol[180] (see Table I) and 24-methylenecholesterol[205] (dienes) are presumably eluted next, followed by ergosterol (triene), which BARRON AND HANAHAN[128] could not resolve from diglycerides.

NEHER's review[101] gives useful information about more complex steroids.

Diglycerides

Since KAUFMANN[7, 9] demonstrated the inverse order of adsorption of simple glycerides, tri- > di- > mono-, diglycerides have quite often been isolated chromatographically[14, 21, 67, 128, 186, 188, 189, 204, 206, 207]. They can be eluted with 20–60% ether in ligroin or with benzene–chloroform mixtures[21, 188]. Resolution of the diglyceride peak after the cholesterol peak is impaired by column overloading[67, 128].

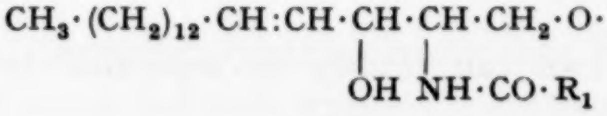
It is reassuring (contrast monoglycerides) that D- and L-1,2-diolein and their tetrabromides[206], and also D-1,2-dicaproin[207], are not isomerized on silicic acid columns. Fractionation of diglycerides seems feasible since distearin tends to be eluted before dipalmitin and dilaurin[67], and 1,3-dipalmitin moves slightly faster than 1,3-diolein on silicic acid-impregnated glass fibre paper (Table I).

Monoglycerides

Most monoglycerides can be eluted with ether or chloroform[21, 67, 128, 138, 186, 188, 190, 204], but monoacetin requires a more polar eluent[67]. 2-Monoglycerides tend to be converted to 1-monoglycerides on silicic acid columns[21, 67]: thus no separation of the isomers is possible.

*Glycolipids**

In 1956 WEISS[95] first reported silicic acid chromatography of cerebrosides which he eluted with a continuous, convex concentration gradient of methanol in chloroform. It seems clear that kerasin (III, $R_1 = (CH_2)_{22}\cdot CH_3$, R_2 = galactose residue) is eluted by chloroform alone[95, 96, 208, 209] and phrenosin (III, $R_1 = CH(OH)\cdot(CH_2)_{21}\cdot CH_3$,



$$CH_3\cdot(CH_2)_{12}\cdot CH{:}CH\cdot\underset{\displaystyle OH}{\underset{|}{CH}}\cdot\underset{\displaystyle NH\cdot CO\cdot R_1}{\underset{|}{CH}}\cdot CH_2\cdot O\cdot R_2$$

(III)

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* Lipids containing bound sugars.

R_2 = galactose residue) by *ca.* 10% methanol in chloroform[95,96,197,208–210] or by acetone[211]. Phrenosin (sulphatide also[212]) precedes phosphatidylethanolamines and most other phospholipids[126,211–213]. But other, less well characterized cerebrosides[197,210–216] and gangliosides[95,216] are eluted with phospholipids. Cytolipin H (III, $R_1 = \mathrm{CH(OH)\cdot(CH_2)_{21}\cdot CH_3}$, R_2 = glucosylgalactose residue?) is eluted before phosphatidylethanolamines[217,218]. A glucose-containing cerebroside from flies is eluted after phosphatidylethanolamines but before lecithins[111].

According to Cleland and Kennedy[209], "Dihydrosphingolipids are eluted only slightly more rapidly than their unsaturated analogues". Psychosine (galactosylsphingosine) is eluted by *ca.* 20% methanol in chloroform, and DNP-psychosine forms a peak between those of kerasin and phrenosin[209].

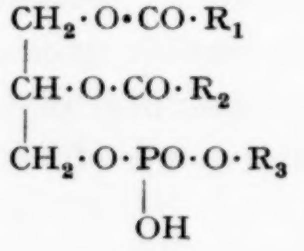
Cord factor from *Mycobacterium tuberculosis* (trehalose 6,6′-dimycolate) is eluted by 20% methanol in ether[61,63] (see also refs.[62,219]) and its methylated derivative by benzene–ether (1:1)[63]. Synthetic compounds related to cord factor have been purified by silicic acid chromatography[64,65], as have other complex glycolipids of mycobacteria[219–223] and plants[150].

Cytidine diphosphate-dipalmitin is eluted by *ca.* 20% methanol in chloroform[224].

Lipoamino-acids[*]

Phosphatidylserines (IV, $R_3 = \mathrm{CH_2\cdot CH(NH_2)\cdot CO_2H}$) are generally eluted in the phosphatidylethanolamine fraction[72,97,98,111–113,150,174,213,226–239].

Marinetti, Erbland and Stotz[85] took a fairly pure sample of phosphatidylserine isolated by non-chromatographic means, and chromatographed it on silicic acid. Eluting with chloroform–methanol (4:1 and 7:3) they obtained two main lipid peaks, the first of which consisted of free phosphatidylserine and the second of its sodium salt; these gave spots with identical R_F values when developed with an acidic solvent on silicic acid-impregnated filter paper. Apparent tailing of phosphatidylserines on columns[97,115,227,230,234–237,239] can be ascribed to this phenomenon. Under certain conditions it appears that silicic behaves as a cation exchanger, liberating phosphatidylserines from their salts[83]. Since phosphatidylserines can readily be separated from other phospholipids on silicic acid-impregnated filter paper (*e.g.* see ref.[240]) it seems that a good separation by column chromatography may sometime be achieved.



$$\begin{array}{l} \mathrm{CH_2\cdot O\cdot CO\cdot R_1} \\ | \\ \mathrm{CH\cdot O\cdot CO\cdot R_2} \\ | \\ \mathrm{CH_2\cdot O\cdot PO\cdot O\cdot R_3} \\ \qquad\quad | \\ \qquad\quad \mathrm{OH} \end{array}$$

(IV)

DNP-phosphatidylserines can readily be separated from DNP-phosphatidylethanolamines[241].

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[*] For definition see ref.[225].

The nature of tissue lipoamino-acids other than phosphatidylserines is still obscure[225], but it is very striking that they are found in minor quantities in most fractions eluted after cholesterol[98, 111–113, 150, 215, 220, 225, 228, 242–247]. Some are eluted by chloroform or ether without added methanol[98, 112, 222, 225, 247].

A synthetic phosphatidyltripeptide (IV, $R_1 = R_2 = (CH_2)_{16}\cdot CH_3$, $R_3 = CH_2\cdot CH(NH_3^+)\cdot CO\cdot NH\cdot CH_2\cdot CO\cdot NH\cdot CH_2\cdot CO_2^-$)[248]* is eluted specifically by 25% methanol in chloroform[115], and its derivative containing phenyl, benzyl and benzyloxycarbonyl protecting groups is eluted by ether[248].

Bile acids

Bile acids have been detected in eluates of blood[112] and liver[128] lipids. Hirsch and Ahrens[67] eluted lithocholic acid in the monoglyceride fraction, followed by desoxycholic acid. Wooton[249] has described chromatography of bile acid methyl esters.

Glycerophosphatidic acids

Phosphatidic acids (IV, $R_3 = H$) have a high R_F value on silicic acid-impregnated filter paper[240, 250–252], and are presumably eluted from columns before phosphatidylethanolamines[227, 253]. Similar behaviour is shown by the ill-characterized polyglycerophosphatidic acids[111, 234–238, 244, 254–257], including cardiolipin[182, 192, 258], although some may be eluted later[238]. Vilkas and Lederer[221] have eluted a mycobacterial phosphatidic acid as its magnesium salt.

Inositol-containing lipids

Phosphatidylinositols (IV, R_3 = 1-inosityl) can be eluted, apparently in salt form, after phosphatidylethanolamines[72, 84, 97, 114, 234, 235, 238, 239, 246, 255, 259, 260]. However, inositol phospholipids have been found eluted before or with phosphatidylethanolamines[150, 182, 228, 237, 261, 262] or as late as lecithins[98, 111, 112, 261]. This suggests the existence of new lipids, and it may indicate that free phosphatidylinositols are eluted earlier than the salts (*cf.* phosphatidylserines).

Phosphatidylethanolamines

Phosphatidylethanolamines (IV, $R_3 = CH_2\cdot CH_2\cdot NH_2$) are most commonly eluted with *ca.* 20% methanol in chloroform[24, 84, 97, 110, 182, 211, 226, 228, 234–239, 255, 263–267] (although methanol concentrations of 15% or below are sufficient[57, 72, 98, 111, 112, 150, 268]), and occasionally with 25% ethanol in hexane[229], 25% methanol in ether[92], or dichloromethane–methanol (13:7)[174]. Rhodes and Lea[226] and Wallach *et al.*[110] have clearly shown that the phosphatidylethanolamines first eluted are the most unsaturated. The reason for this behaviour, which is contradistinct from that of non-phospholipids, is not known; perhaps it reflects the degree of unsaturation in only one fatty acid residue (IV, R_1 or R_2).

Synthetic L-α-(dioleyl)-phosphatidylethanolamine has been eluted with chloroform–methanol (4:1), and its phthalimide with ether–methanol (4:1)[267].

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* A sample was generously provided by Professor E. Baer.

Lysophosphatidylethanolamines

Lysophosphatidylethanolamines are eluted just before[111, 115, 182, 237, 265] and with[111, 115, 182, 226, 265] lecithins.

Lecithins

Lecithins (phosphatidylcholines; IV, $R_3 = CH_2 \cdot CH_2 \cdot N^+(CH_3)_3{}^-OH$) can be eluted slowly with as little as 20% methanol in chloroform[98, 115, 226, 269], but usually higher methanol concentrations are employed[24, 67, 72, 84, 97, 98, 111, 112, 150, 182, 194, 211, 230, 231, 234, 235, 238, 239, 234, 265, 268, 270–273] and occasionally mixtures of other liquids[174, 228, 229, 263, 274, 275]. Many workers[112, 174, 211, 230, 231, 234, 235, 238, 239, 262, 264, 268, 276, 277] have found it difficult to resolve the lecithin peak from the sphingomyelin peak that follows, but it is possible[57, 67, 72, 97, 182, 228, 263, 265, 273], and is facilitated by continuous concave gradient elution[98]. This method prevents the tailing characteristic of tissue lecithins. On silicic acid-impregnated glass fibre paper L-α-(dipalmitoleyl)-lecithin moves faster than L-α-(distearyl)-lecithin (see Table I; but note that a mixture could not be resolved), and the most unsaturated lecithins are eluted first from columns[97, 226] (*cf.* phosphatidylethanolamines).

Sphingomyelins

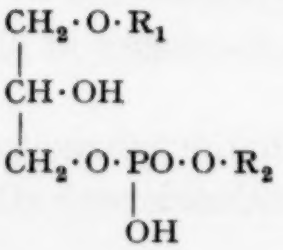
Sphingomyelins (III, $R_2 = O \cdot PO(O^-) \cdot O \cdot CH_2 \cdot CH_2 \cdot N^+(CH_3)_3$) can be separated in a peak between lecithins and lysolecithins[228, 230, 231, 278]. A concave concentration gradient of 30–50% methanol in chloroform elutes them[98].

Lysolecithins

Eluents used for lysolecithins range in polarity[24, 226, 230, 231, 235, 236, 239, 271] from 50% methanol in chloroform[265, 269] to methanol containing water[228, 263, 278]. Apparently silicic acid catalyzes acyl migration, 2-(acyl)-lysolecithins changing to the 1-[195, 265, 279], although TATTRIE AND MCARTHUR[269] may have separated 1- and 2-(palmityl)-lysolecithins, eluting the 2- first. On silicic acid-impregnated glass fibre paper (Table I) 2-(palmitoleyl)- moves faster than 2-(stearyl)-lysolecithin.

Ethers

"If an ether linkage replaces one of the carboxyl ester linkages in a triglyceride, the pattern of elution is unchanged"[67]. Thus, compounds such as chimyl dioleate are eluted with triglycerides[67, 138, 185]. Ligroin–ether (17:3) elutes the benzyl ether of D-1,2-dicaproin[207]. Chimyl (hexadecyl) and batyl alcohols (octadecyl D-1-glyceryl ether) are eluted with monoglycerides[67, 140, 185, 194]; 2-glyceryl ethers are more readily eluted than 1-glyceryl ethers[195, 266]. Glyceryl-1-phosphate-3-ethers (V, $R_2 = H$) can

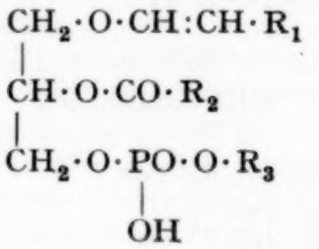


$$\begin{array}{l} CH_2 \cdot O \cdot R_1 \\ | \\ CH \cdot OH \\ | \\ CH_2 \cdot O \cdot \underset{\displaystyle OH}{\underset{|}{PO}} \cdot O \cdot R_2 \end{array}$$

(V)

be eluted with 20% methanol in chloroform (chloroform alone is insufficiently polar) and the ether analogue of lysophosphatidylethanolamine (V, $R_2 = O\cdot CH_2\cdot CH_2\cdot NH_2$) with 55% methanol[197,210].

It has not yet been possible to separate ethanolamine plasmalogens (VI, $R_3 = CH_2\cdot CH_2\cdot NH_2$) from phosphatidylethanolamines[57,81,98,111,112,182,194,214,266,280] or choline plasmalogens (VI, $R_3 = CH_2\cdot CH_2\cdot N^+(CH_3)_3{}^-OH$) from lecithins[57,98,111,112,182,194,234,239,265,266,273,280]. It is claimed that the former separation can be achieved



$$\begin{array}{l} CH_2\cdot O\cdot CH{:}CH\cdot R_1 \\ | \\ CH\cdot O\cdot CO\cdot R_2 \\ | \\ CH_2\cdot O\cdot PO\cdot O\cdot R_3 \\ \qquad\quad\;\, | \\ \qquad\quad\; OH \end{array}$$

(VI)

on silicic acid-impregnated filter paper at low temperature[236]. Ethanolamine lysoplasmalogens can be eluted with 50% ethanol in hexane[81].

Being vinyl ethers, plasmalogens are very reactive in presence of catalytic quantities of acids or heavy metal ions. Thus it is not unreasonable to suspect losses by hydrolysis during chromatography. GRAY AND MACFARLANE[182] showed indeed that some plasmalogen was hydrolyzed when a sample of whole ox-heart lipid was chromatographed in chloroform, followed by chloroform–methanol. After this experiment the column was exhaustively washed with chloroform and a second sample of the same lipid was chromatographed on it: plasmalogen was not then hydrolyzed. When material eluted by chloroform–methanol was introduced to a new column, in chloroform–methanol solution, hydrolysis was not detected. The interpretation made of these findings was that limited hydrolysis of plasmalogens occurs on silicic acid only in presence of the "fat" fraction. Another interpretation, not excluded by the findings reported, is that silicic acid catalyzes hydrolysis before, but not after, contact with methanol.

Miscellaneous lipids

α-Tocopherol[67,92,152,281] and its acetate[92] are eluted at about the same stage as cholesteryl esters (see Table I) but after the synthetic antioxidant 2,6-di-*tert.*-butyl-*p*-cresol[282]. Numerous pigments of animal[98,110,174,232,283] and plant[150,284] tissues are eluted before phosphatidylethanolamines. Propylene glycol mono- and distearates can be eluted with chloroform[285], bis(distearyl-L-1-glyceryl) phenyl phosphate with ligroin–ether (4:1)[248], ceramides (III, R_2 = H) with 5% methanol in chloroform[112,197,210], sphingosine with chloroform–methanol (*ca.* 10:1)[209,286], DNP-sphingosine with > 30% chloroform in benzene[209], and sphingosine hydrochloride with methanol[155].

Finally, some lipids of unproved structure will be mentioned. A primary aromatic palmitamide derivative in the unsaponifiable fraction of human liver has been isolated by elution with 40% ether in pentane[140]. Sirenin ($C_{21}H_{36}O_7$?), the sex-attractant of

Allomyces, is eluted by chloroform–methanol (99:1)[287, 288]. An "unnaturally biosynthesized" diethanolamine lipid is eluted at the same stage as phosphatidylethanolamines[289]. N-Mono- and N,N-dimethylethanolamine lipids of *Neurospora* are eluted in the phospholipid fractions[290].

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QUANTITATIVE INORGANIC CHROMATOGRAPHY

PART VI. APPLICATION TO KINETIC STUDIES: THE OXIDATION OF HYDRAZINE BY FERRIC IRON IN ACID AQUEOUS SOLUTIONS

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The use of ion-exchange and/or paper chromatography to elucidate the mechanism of inorganic reactions has not, up to the present, been extensively used, though there are clearly fields where its application might do much to establish the correctness of postulates made from the classical physicochemical approach, or to throw light on hitherto unsuspected intermediates or products which would not be revealed by classical analytical procedures. A number of reactions have been studied to show how chromatography can help in this field, and this paper represents the first of such investigations.

When hydrazine is oxidised in aqueous solution, the principal nitrogenous products are ammonia, nitrogen and hydrazoic acid[1]. The proportions of these products vary considerably with the oxidising agent and the conditions of the reaction. In a paper summarising much of the early work on the oxidation of hydrazine, KIRK AND BROWNE[2] pointed out a number of common features, and two main classes of oxidising agent were distinguished:

(i) those capable of producing hydrazoic acid, ammonia and nitrogen,

(ii) those incapable of producing hydrazoic acid.

Ferric iron in acid solution oxidises hydrazine and belongs to the second class, and the overall equation may be written:

$$N_2H_5^+ \rightarrow e + NH_4^+ + \tfrac{1}{2}N_2 + H^+$$

The stoichiometry of the reaction (*i.e.* the change in equivalent concentration of oxidising agent divided by the corresponding change in molecular concentration of hydrazine) is approximately equal to 1.3.

CUY[3] proposed that the reaction proceeded via the intermediate N_2H_3, the hydrazyl radical, which he termed an odd molecule[1]; it contains an uneven number of electrons. KIRK AND BROWNE[2] suggested that the following hypothetical mechanism was adequate to account for the products obtained with class (ii) oxidising agents.

$$N_2H_4 \xrightarrow{\text{1 equiv.}} N_2H_3 \rightarrow \tfrac{1}{2}N_4H_6\ (H_2N\cdot NH\cdot NH\cdot NH_2)$$

$$2N_2H_3 \rightarrow N_4H_6 \rightarrow 2NH_3 + N_2$$

$$N_4H_6 \xrightarrow{\text{2(1 equiv.)}} H_2N\cdot N{:}N\cdot NH_2 \rightarrow N_2H_4 + N_2$$

Two independent kinetic investigations[4, 5] of the slow reaction between iron(III) and hydrazine in acid solution have been carried out to elucidate the mechanism of the reaction, but slightly different conclusions were reached. CAHN AND POWELL[4] conclude that the reaction

$$2N_2H_3 \rightarrow N_2H_4 + N_2H_2$$

competes with the reaction

$$2N_2H_3 \rightarrow N_4H_6$$

whilst HIGGINSON AND WRIGHT[5] believe the disproportionation reaction to be of minor importance.

The reaction mechanisms proposed by the two groups of workers are:

$$\text{Fe(III)} + N_2H_4 \xrightarrow{k_1} \text{Fe(II)} + N_2H_3 \qquad (1)$$

$$\text{Fe(II)} + N_2H_3 \xrightarrow{k_{-1}} \text{Fe(III)} + N_2H_4 \qquad (-1)^*$$

$$2N_2H_3 \xrightarrow{k_2} N_4H_6 \qquad (2)$$

followed by

$$N_4H_6 \rightarrow 2NH_3 + N_2$$

$$2N_2H_3 \xrightarrow{k_3} N_2H_4 + N_2H_2 \qquad (3)^{**}$$

followed by

$$N_2H_2 \xrightarrow[\text{rapid}]{2(1\ \text{equiv.})} N_2$$

$$\text{Fe(III)} + N_2H_3 \xrightarrow{k_4} \text{Fe(II)} + N_2H_2 \qquad (4)$$

followed by

$$N_2H_2 \xrightarrow[\text{rapid}]{2(1\ \text{equiv.})} N_2$$

HIGGINSON AND WRIGHT[5] measured the rate of production of ferrous iron colorimetrically with *o*-phenanthroline and the ferric iron remaining spectrophotometrically as the sulphate complex at 303 mμ. CAHN AND POWELL[4] determined the overall stoichiometry of the reaction only; by employing a large excess of the oxidising agent, the ferrous iron produced was determined by titration with potassium permanganate.

The disagreement between the two mechanisms is because HIGGINSON AND WRIGHT[4] state that the reaction (— 1) must be taken into account. If this is so, when hydrazyl radicals or ferrous ions are removed from the reaction scheme, then reaction (— 1) may be neglected. CAHN AND POWELL[4] showed that cupric copper increases the stoichiometry of the oxidation of hydrazine by ferric iron to 3.9 and ascribe this to the reactions

$$N_2H_3 + \text{Cu(II)} \rightarrow \text{Cu(I)} + N_2H_2 \qquad (5a)$$

$$\text{Cu(I)} + \text{Fe(III)} \rightarrow \text{Cu(II)} + \text{Fe(II)} \qquad (5b)$$

since cupric copper does not oxidise hydrazine alone. Thus, if reaction (— 1) is important, the rate of disappearance of hydrazine will be considerably increased in the

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[*] HIGGINSON AND WRIGHT'S mechanism only.

[**] CAHN AND POWELL'S mechanism only.

presence of Cu(II), since reaction (5a) will decrease the stationary concentration of hydrazyl radicals and hence the extent of reaction (— 1). Whilst the present investigation was in progress, ROSSEINSKY[6] reported that the rate of disappearance of hydrazine was increased by the presence of Cu(II), and thus independent evidence for the importance of reaction (— 1) has been obtained.

EXPERIMENTAL

All water was redistilled and stored in Jena glass aspirators. 99 % hydrazine hydrate (B.D.H.) was standardised by titration with potassium iodate solution using the normal Andrew's conditions. Stock hydrazine solutions of varying molarities were prepared by dilution of the hydrazine hydrate with either 1 *M* hydrochloric acid or 0.5 *M* sulphuric acid. Iron(II) chloride and iron(II) sulphate solutions were prepared by dissolving weighed amounts of pure iron wire in excess of hydrochloric or sulphuric acids respectively. Iron(III) chloride or iron(III) sulphate solutions were prepared by oxidation of the corresponding iron(II) solutions with 100 volume hydrogen peroxide, the excess peroxide was destroyed by gentle heating for some hours. Iron(II) or iron(III) concentrations were determined in these stock solutions by titration with potassium dichromate in dilute sulphuric acid or gravimetrically as the oxide respectively. The iron solutions were outgassed with nitrogen and sealed until used. All other stock solutions were prepared from AnalaR reagents where possible, and the concentrations determined by appropriate methods.

In the kinetic experiments in chloride solutions, a constant initial hydrogen ion concentration was essential, and it was necessary to know the second dissociation constant of hydrazine,

$$K_2 = \frac{[N_2H_5^+]\,[H^+]}{[N_2H_6^{2+}]}$$

since the initial hydrazine concentration was varied from 0.242–0.024 *M* in the investigation. The value of K_2 was assumed to be 6 ± 2 moles·l^{-1} (see HIGGINSON AND WRIGHT[5]).

The initial hydrogen ion concentration in sulphate solutions was calculated by assuming that sulphuric acid was completely dissociated. This is not true, but the variation of K_2 for sulphuric acid with ionic strength of the solution is such as to render the calculation of the exact hydrogen ion concentration very difficult[7].

The reactions were carried out in a thermostat at either 25° ± 0.1°, or 50° ± 0.25°. A mixture of all reagents except the appropriate hydrazine stock solution was outgassed with nitrogen and allowed to come to equilibrium in the thermostat. The reaction was started by addition of the hydrazine solution. Samples were withdrawn for ammonia and iron analyses within a few seconds of mixing. As the reaction proceeded, samples were removed for analysis when convenient, *i.e.* for reactions at 25° every 2 hours, and every hour when at 50°.

Ammonia was estimated in the following manner. In one of the samples, hydrazine was destroyed by addition of a small excess of acid potassium iodate, and the iodine produced was destroyed by sodium sulphite. Excess sulphite was destroyed by boiling. The solution was transferred to a MARKHAM still[8] (with ground glass joints) and made alkaline with 10 N caustic soda solution. The ammonia was distilled off into 2 % w/v boric acid solution and titrated with 0.01 M hydrochloric acid using a mixed methyl red–methylene blue indicator[9].

Iron(II) and iron(III) were estimated using the anion-exchange and colorimetric procedure described by POLLARD, McOMIE, NICKLESS AND HANSON[10]. The paper chromatographic separation[11] was also employed but iron(III) trailed backwards towards the starting line, making quantitative results impossible. The probable reason for this is that hydrazine accompanies the iron(III) up the paper and iron(II) is formed by continued reaction between the two ionic species. As iron(II) has a low R_F value in the solvent used, it is left behind by the solvent, thus forming a long streak on the paper. In the anion-exchange procedure, however, hydrazine (as $N_2H_5^+$) is unadsorbed and accompanies the iron(II).

The iron(II) results were checked by measurement of the optical density at 510 mμ of the tris-*o*-phenanthroline complex in citrate buffer[12]. Iron(III) results were checked by the measurement of change in optical density at 620 mμ of the Ferron complex at pH 2.6.[13] Iron(II), and iron(III) may be determined to an accuracy of ± 2 %, and ammonia to within ± 3 %.

Plots of the iron(II) and ammonia concentrations against time were made from these results and the gradients found at times given in the Tables below.

$$R(\mathrm{NH_3}) = \frac{\mathrm{d}\,[\mathrm{NH_3}]}{\mathrm{d}t} \qquad \text{and} \qquad R(\mathrm{Fe}) = \frac{\mathrm{d}\,[\mathrm{Fe(II)}]}{\mathrm{d}t}$$

were estimated to be within ± 5 %.

The rate of disappearance of hydrazine was observed by measuring the change in optical density at 455 mμ of the azine formed between hydrazine and *p*-dimethylaminobenzaldehyde[14, 15] in dilute hydrochloric acid solution This method was considered more accurate than the procedure described by ROSSEINSKY[6], since iron(II), iron(III) or copper(II) did not interfere.

RESULTS AND DISCUSSION

In agreement with earlier mechanisms, hydrazine and the derived radicals are depicted as possessing zero charge. Almost certainly this is not the case, but there is no evidence necessitating that the radicals should react in a particular form. The formulae used are merely to represent the oxidation state of the nitrogen atoms concerned and not the detailed structure of the radical.

Fig. 1 shows a plot of

$$\log_{10} \frac{[\mathrm{Fe(III)}]_t}{[\mathrm{N_2H_4}]_t} \frac{[\mathrm{N_2H_4}]_0}{[\mathrm{Fe(III)}]_0}$$

against time (in hours) for a reaction mixture composed of $[\mathrm{Fe(III)}]_0 = 0.100\ M$, and $[\mathrm{N_2H_4}] = 0.094\ M$ in chloride solutions at 25°.

Effect of ferrous iron

When increasing amounts of ferrous chloride are added to such a reaction mixture as above, deviations from bimolecularity are immediately apparent. As the concentration of ferrous iron is increased, so the deviation increases, *viz.* the reaction becomes slower, indeed if the ratio $[\mathrm{Fe(II)}]_0/[\mathrm{Fe(III)}]_0$ is > 3, the reaction almost ceases (see Fig. 1).

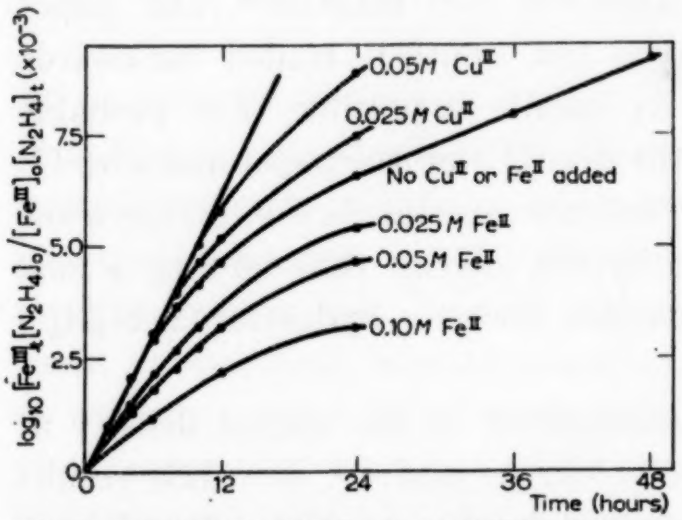

Fig. 1. The effect of time and added salts upon the initial reaction rate in chloride solutions at 25°.

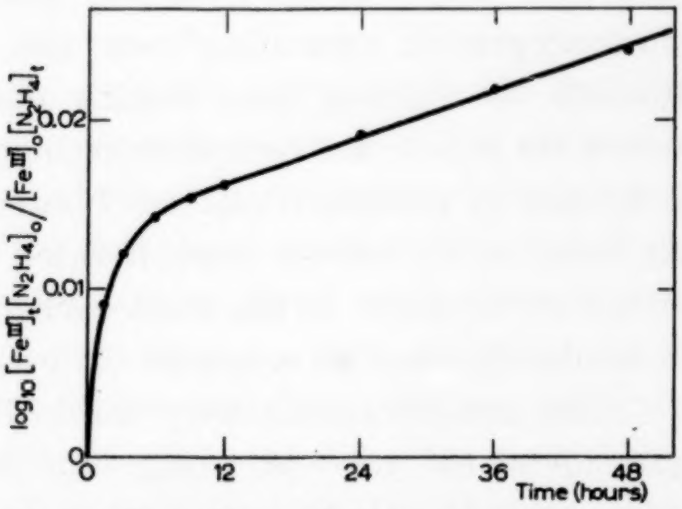

Fig. 2. The effect of time upon the initial reaction rate in sulphate solutions at 25°.

Effect of cupric copper

When copper(II) chloride is added to the reaction solutions, then the deviation from bimolecularity is less than when copper(II) is absent (see Fig. 1). As the copper(II) concentration is increased, the deviation at any given time becomes less than when copper(II) is absent. This effect is probably due to copper(II) removing hydrazyl radicals from the reaction scheme (see eqn. 5a) faster than does iron(III).

Reaction velocity

The variation in velocity constant of reaction (1) with time, assuming that reaction (— 1) may be neglected, is given in Table I.

TABLE I

Time h	*Velocity k_1 (in $l \cdot moles^{-1} \cdot h^{-1}$)*			
	No Fe(II)	*0.025 M Fe(II)*	*0.050 M Fe(II)*	*0.100 M Fe(II)*
2	0.197	—	—	—
4	0.197	0.180	0.120	—
6	0.196	0.137	0.113	0.075
8	0.173	0.129	0.109	—
10	0.159	0.122	0.105	—
12	0.147	0.117	0.102	0.068
24	0.105	0.086	0.057	0.043
36	0.083	—	—	—
48	0.073	—	—	—

HIGGINSON AND WRIGHT[5] quote a value of $k_1 = 0.06$ l·moles^{-1}·h^{-1} for low ferrous iron concentrations at 25°; unfortunately this has very little meaning unless the ferrous iron concentration is also quoted.

The results for sulphate-containing solutions are very similar and a plot of

$$\log_{10} \frac{[\text{Fe(III)}]_t}{[\text{N}_2\text{H}_4]_t} \frac{[\text{N}_2\text{H}_4]_0}{[\text{Fe(III)}]_0}$$

against time is given in Fig. 2. Retardation by ferrous iron is much greater than in chloride solutions. The velocity of reaction (1), k_1 varies from 1.36 l·moles^{-1}·h^{-1} with no iron(II) present to 0.16 l·moles^{-1}·h^{-1} after 48 h (*i.e.* 20 % reaction).

Quantitative investigation of the reaction mechanism

At 25° the ammonia analyses are insufficiently accurate, over the range of stoichiometries investigated, to permit an unambiguous kinetic interpretation. Consequently, the temperature was raised to 50° to enable the reaction to proceed at a faster rate.

From the proposed reaction mechanism, and with Cu(II) absent

$$R(\text{NH}_3) = k_2 [\text{N}_2\text{H}_3]^2 \tag{6}$$

$$R(\text{Fe}) = k_1 [\text{Fe(III)}][\text{N}_2\text{H}_4] - k_{-1} [\text{Fe(II)}][\text{N}_2\text{H}_3] + k_3 [\text{N}_2\text{H}_3]^2 + k_4 [\text{Fe(III)}][\text{N}_2\text{H}_3] \tag{7}$$

where $R(\text{NH}_3) = \text{d}\,[\text{NH}_3]/\text{d}t$; and $R(\text{Fe}) = \text{d}\,[\text{Fe(II)}]/\text{d}t$.

Making the normal stationary state assumption that

$$\begin{aligned} 0 = \text{d}\,[\text{N}_2\text{H}_3]/\text{d}t = k_1 [\text{Fe(III)}][\text{N}_2\text{H}_4] - k_{-1} [\text{Fe(II)}][\text{N}_2\text{H}_3] - k_2 [\text{N}_2\text{H}_3]^2 \\ - k_3 [\text{N}_2\text{H}_3]^2 - k_4 [\text{N}_2\text{H}_3][\text{Fe(III)}] \end{aligned} \tag{8}$$

Subtract (8) from (7)

$$R(\text{Fe}) = k_2 [\text{N}_2\text{H}_3]^2 + 2k_3 [\text{N}_2\text{H}_3]^2 + 2k_4 [\text{Fe(III)}][\text{N}_2\text{H}_3] \tag{9}$$

Divide (9) by (6)

$$R(\text{Fe})/R(\text{NH}_3) = 1 + 2k_3/k_2 + 2k_4 [\text{Fe(III)}]/k_2 [\text{N}_2\text{H}_3] \tag{10}$$

Eliminating $[\text{N}_2\text{H}_3]$ by means of (6)

$$R(\text{Fe})/R(\text{NH}_3) = 1 + 2k_3/k_2 + 2k_4 [\text{Fe(III)}]/\sqrt{k_2 R(\text{NH}_3)} \tag{11}$$

Thus a plot of $R(\text{Fe})/R(\text{NH}_3)$ against $[\text{Fe(III)}]/\sqrt{R(\text{NH}_3)}$ should be a straight line of intercept $1 + 2k_3/k_2$. The intercept is nearly unity, and was evaluated by rewriting (11) in the form

$$\frac{R(\text{Fe})}{[\text{Fe(III)}]\sqrt{R(\text{NH}_3)}} = \left(1 + \frac{2k_3}{k_2}\right) \frac{\sqrt{R(\text{NH}_3)}}{[\text{Fe(III)}]} + \frac{2k_4}{\sqrt{k_2}} \tag{12}$$

A plot of the left-hand side of eqn. (12) against $\sqrt{R(\text{NH}_3)}/[\text{Fe(III)}]$ is shown in Fig. 3 for experiments at 50° in chloride solutions, the data for which are summarised in Table II. All rates were measured by drawing the tangent to the concentration *versus* time curves at $t = 0$.

TABLE II

CHLORIDE SOLUTIONS AT 50°

$[Fe(II)]_0 = 0$

Experiment No.	*[Fe(III)]* *moles·l⁻¹*	*[N₂H₄]* *moles·l⁻¹*	*R(Fe)* *moles·l⁻¹·h⁻¹*	*R(NH₃)* *moles·l⁻¹·h⁻¹*	$\frac{\sqrt{R(NH_3)}}{[Fe(III)]}$	$\frac{R(Fe)}{[Fe(III)]\sqrt{R(NH_3)}}$
1	0.100	0.094	0.0194	0.0187	1.379	1.412
2	0.100	0.047	0.0125	0.0126	0.122	1.100
3	0.100	0.019	0.0047	0.0048	0.691	0.690
4	0.050	0.098	0.0116	0.0115	2.150	2.165
5	0.020	0.098	0.0056	0.0052	3.606	3.632
6	0.050	0.049	0.0063	0.0051	1.583	1.606

Fig. 3 gives a gradient of $1 + 2k_3/k_2 = 1.025 \pm 0.02$, whence

$$k_3/k_2 = 0.012 \pm 0.010\ .$$

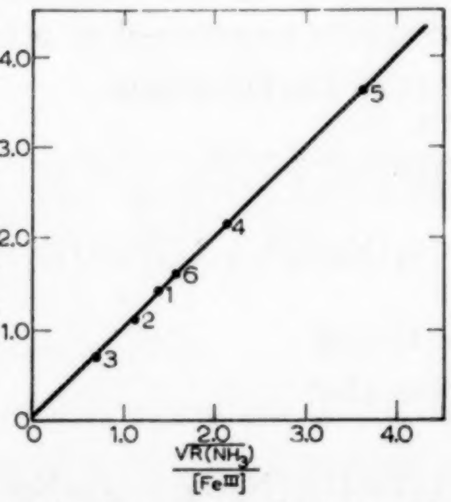

Fig. 3. The test of equation (12) in chloride solutions at 50°.

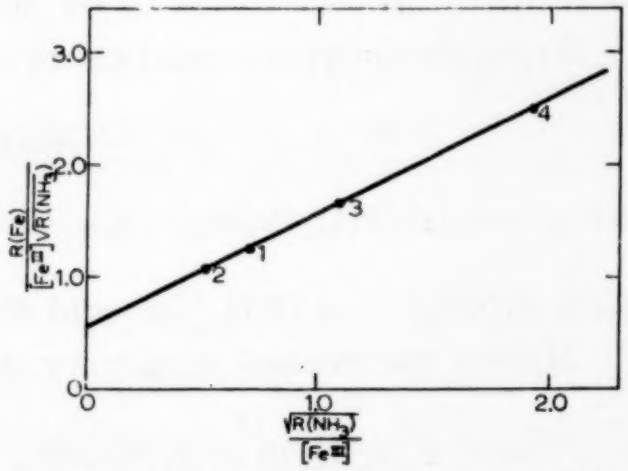

Fig. 4. The test of equation (12) in sulphate solutions at 50°.

The results for sulphate solutions at 50° are shown in Fig. 4 from experiments summarised in Table III.

TABLE III

SULPHATE SOLUTIONS AT 50°

$[Fe(II)]_0 = 0$

Experiment No.	*[Fe(III)]* *moles·l⁻¹*	*[N₂H₄]* *moles·l⁻¹*	*R(Fe)* *moles·l⁻¹·h⁻¹*	*R(NH₃)* *moles·l⁻¹·h⁻¹*	$\frac{\sqrt{R(NH_3)}}{[Fe(III)]}$	$\frac{R(Fe)}{[Fe(III)]\sqrt{R(NH_3)}}$
1	0.100	0.0268	0.0088	0.0052	0.721	1.250
2	0.100	0.0134	0.0062	0.0027	0.519	1.083
3	0.050	0.0268	0.0048	0.0030	1.096	1.650
4	0.020	0.0268	0.0020	0.0013	1.970	2.560

Whence $$k_3/k_2 = 0.015 \pm 0.015 \quad \text{at } 50°\ .$$

These values are to be compared with CAHN AND POWELL'S[4] result of 0.15 at 50°, but they neglected the back reaction (—1) in their mechanism. HIGGINSON AND WRIGHT[5] report that at 60°

$$k_3/k_2 = 0.015 \pm 0.015\ .$$

The linear plots obtained, provide good evidence that the reaction scheme proposed by HIGGINSON AND WRIGHT[5] is valid.

Effect of high concentrations of ferrous iron

At high concentrations of ferrous iron, the rate of disappearance of hydrazyl radicals by reactions (2), (3) and (4) will be negligible as compared to back reaction (— 1).

Then as an approximation, if

$$K = k_1/k_2$$

$$[N_2H_3] = K\,[Fe(III)]\,[N_2H_4]/[Fe(II)] \tag{13}$$

From (13) and (6)

$$R(NH_3) = k_2K^2\,[Fe(III)]^2\,[N_2H_4]^2/[Fe(II)]^2 \tag{14}$$

from (13) and (10)

$$R(Fe)/R(NH_3) = 1 + 2k_3/k_2 + 2k_4\,[Fe(II)]/Kk_2\,[N_2H_4] \tag{15}$$

Thus for equation (14) a plot of $R(NH_3)$ *versus* $[Fe(III)]^2\,[N_2H_4]^2/[Fe(II)]^2$ will be a straight line passing through the origin. Similarly for equation (15) a plot of $R(Fe)/R(NH_3)$ against $[Fe(II)]/[N_2H_4]$ should be a straight line, of intercept $1 + 2k_3/k_2$, whence k_3/k_2 may be deduced.

Plots for equations (14) and (15) are shown in Figs. 5 and 6 respectively, from experiments at 50° in chloride solutions, which are summarised in Table IV.

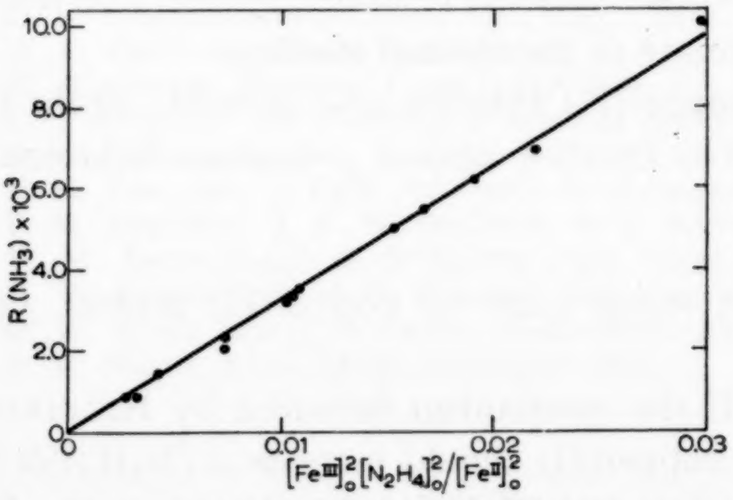

Fig. 5. The test of equation (14) in chloride solutions at 50°; where reaction (— 1) ≫ reactions (2, 3, or 4).

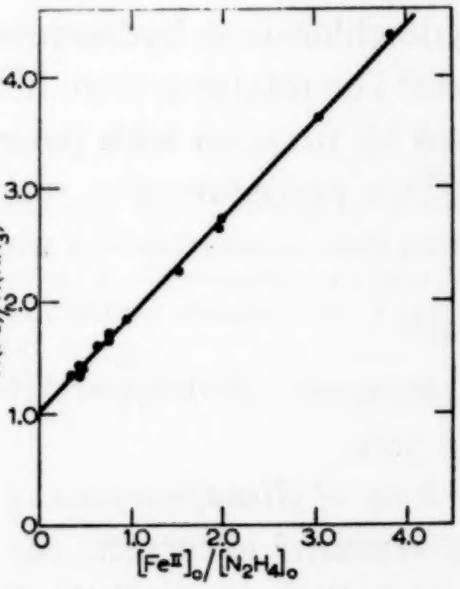

Fig. 6. The test of equation (15) in chloride solutions at 50°.

From Fig. 6, $k_3/k_2 = 0.025 \pm 0.015$ which is in excellent agreement with the value of k_3/k_2 calculated using equation (12).

It was thought that the linearity of these plots, *i.e.* Figs. 5 and 6, gave additional evidence for the validity of the proposed reaction mechanism.

Effect of cupric copper

Stoichiometry. It has already been shown (Fig. 1), that the presence of copper(II) tends to keep the deviations from bimolecularity smaller than if copper(II) is absent.

To account for this, it has been postulated[4] that copper(II) removes the hydrazyl radicals through equation (5a), faster than does iron(III) by reaction (4). Hence, the amount of 4-electron oxidation must increase *i.e.* the stoichiometry when copper(II) is absent must be less than when copper(II) is present.

TABLE IV

CHLORIDE SOLUTIONS AT 50°

Experiment No.	$[Fe(III)]$ *moles·l*$^{-1}$	$[Fe(II)]$ *moles·l*$^{-1}$	$[N_2H_4]$ *moles·l*$^{-1}$	$\frac{[Fe(III)]^2[N_2H_4]^2}{[Fe(II)]^2}$ *moles*^{2}*·l*$^{-2}$	$R(NH_3)$ *moles·l*$^{-1}$*·h*$^{-1}$	$R(Fe)$ *moles·l*$^{-1}$*·h*$^{-1}$	$\frac{R(Fe)}{R(NH_3)}$	$\frac{[Fe(II)]}{[N_2H_4]}$
1	0.0500	0.0480	0.0976	0.0103	0.00301	0.00425	1.41	0.492
2	0.100	0.0966	0.0438	0.00258	0.00083	0.00225	2.70	1.970
3	0.0800	0.1128	0.2425	0.02958	0.0100	0.01426	1.43	0.465
4	0.0800	0.1128	0.1212	0.00739	0.0020	0.00370	1.84	0.931
5	0.0400	0.1128	0.2425	0.00739	0.0023	0.00340	1.36	0.465
6	0.200	0.0940	0.0485	0.0106	0.00334	0.00909	2.72	1.940
7	0.100	0.0980	0.1488	0.0220	0.00690	0.0110	1.60	0.644
8	0.100	0.0980	0.1212	0.0153	0.00505	0.00863	1.71	0.755
9	0.050	0.0113	0.1950	0.0191	0.00625	0.0102	1.64	0.750
10	0.0800	0.1504	0.1950	0.0109	0.00350	0.00600	1.71	0.771
11	0.200	0.0940	0.0243	0.00265	0.000850	0.00365	4.29	3.86
12	0.200	0.0752	0.0243	0.00417	0.00140	0.00508	3.63	3.09
13	0.200	0.0752	0.0486	0.01670	0.00543	0.0124	2.26	1.54

Experimental. Reaction mixtures containing ferric chloride and hydrazine dihydrochloride in hydrochloric acid were prepared, the ferric chloride being in large excess. The mixtures were heated for 4 h at 90°, and the resultant ferrous iron determined by titration with potassium dichromate in the normal manner.

This procedure was repeated with copper(II) chloride also present; again the ferrous iron concentration was determined by titration against potassium dichromate.

Results

Stoichiometry. (1) Copper(II) absent. 1.25; 1.29; 1.28. (2) Copper(II) present 3.94, 3.90, 3.91.

Rate of disappearance of hydrazine. If the mechanism favoured by HIGGINSON AND WRIGHT[5] is correct, the presence of copper(II) should increase d $[N_2H_4]/dt$ by reducing the extent of the back reaction (— 1). d $[N_2H_4]/dt$ has been measured in the absence of copper(II), and with different amounts of copper(II) present. The results are summarised in Table V.

The results provide clear evidence for the large catalytic effect of copper(II), and hence the back reaction (— 1) must be appreciable, since in similar experiments with iron(III) absent, copper(II) did not oxidise hydrazine.

Initial rates are similar in all the experiments, since in the early stages of the reaction, the back reaction (— 1) is negligible. As the reaction proceeds, and iron(II) accumulates, the difference in the rates in the presence and absence of copper(II) increases, which is in accordance with ROSSEINSKY'S results and the observations expected from HIGGINSON AND WRIGHT'S mechanism.

TABLE V

VALUES OF $d[N_2H_4]/dt$ (moles · l^{-1} · h^{-1}) AT VARIOUS MOLAR CONCENTRATIONS OF COPPER(II)
[HCl] = 1.00 *M*, initial $[N_2H_4]$ = 0.047 *M*, initial [Fe(III)] = 0.100 *M*. Temperature = 50°

Expt. No.	*$[CuCl_2]$ moles·l^{-1}*	*$d[N_2H_4]/dt$ (moles·l^{-1}·h^{-1})*						
		10^2 $[N_2H_4]$ (moles·l^{-1})						
		4.00	*3.75*	*3.50*	*3.00*	*2.50*	*2.00*	*1.75*
1	0	0.015	0.013	0.0084	0.0047	0.0029	0.0020	0.0012
2	0.025	0.015	0.013	0.0084	0.0055	0.0038	0.0028	0.0024
3	0.050	0.015	0.013	0.0087	0.0065	0.0043	0.0034	0.0030
4	0.075	0.015	0.014	0.0090	0.0070	0.0054	0.0040	0.0035

SUMMARY

The mechanism proposed by HIGGINSON AND WRIGHT for the slow reaction between ferric iron and hydrazine in acid aqueous solutions has been confirmed, using an anion-exchange procedure for the separation and quantitative estimation of ferrous and ferric iron.

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COLOUR REACTIONS GIVEN BY SUGARS AND DIPHENYLAMINE-ANILINE SPRAY REAGENTS ON PAPER CHROMATOGRAMS

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INTRODUCTION

A spray reagent containing diphenylamine, aniline and phosphoric acid was developed by BUCHAN AND SAVAGE[1] for locating hexoses on paper chromatograms. SCHWIMMER AND BEVENUE[2] later showed that, with controlled heating at 80° for 5 min, the spray gave a specific colour reaction (bright blue) with disaccharides containing a glycosidic link at position 4 of the reducing monosaccharide unit (*e.g.* maltose, cellobiose and lactose). The colour was in marked contrast to the yellow-brown or grey colours given by other glucose and galactose disaccharides. The spray has since proved of value in identifying these 1,4-linked sugars on chromatograms of oligosaccharide mixtures[3,4]. Under the same conditions the colours given by other glucose disaccharides appeared, in some cases, to be specific for the type of glycosidic link present although colour differences were not so marked[4]. In the case of monosaccharides, SMITH[5] tested the reaction of a number of them with the spray on papers heated at 95–100° for a few minutes. In general grey-brown or brown colours were obtained. Exceptions were D-glucose and D-galactose (blue), L-sorbose and D-galacturonic acid (green).

In view of the results reported for the glucose disaccharides it seemed likely that, under more moderate heating conditions, other specific colour reactions with various types of monosaccharides might be obtained. A detailed study of the reaction of the spray with a comprehensive collection of sugars has, therefore, been made. The results obtained, which are reported here, considerably extend the range of specific colour reactions given by the spray. As a result of attempts to obtain an indication of the nature of the reactions involved, two modified versions of the spray, giving different or better colour reactions, have been developed.

EXPERIMENTAL

Materials

All of the sugars used were obtained from commercial sources or the departmental collection, except for xylobiose (4-O-β-D-xylopyranosyl-D-xylopyranose), rhodymenabiose (3-O-β-D-xylopyranosyl-D-xylopyranose), digalacturonic acid (4-O-β-D-galactopyruronosyl-D-galacturonic acid) and L-gulose. These were supplied by Dr. B. H. HOWARD (Rowett Research Institute, Aberdeen), Mr. W. W. REID (Carreras Ltd., London) and Dr. D. H. HUTSON (of this laboratory), respectively. Most of the sugars were chromatographically pure; in other cases the solvents used for developing chromatograms clearly separated the sugar under study from any contaminating sugars.

Furfural (B.D.H. Technical) was distilled under reduced pressure to give a water-white product which was kept at 0°. Purified 5-hydroxymethylfurfural was supplied by Dr. M. RUSZKIEWICZ of this laboratory.

The components of the sprays were B.D.H. reagent-grade chemicals which were not further purified.

Chromatograms

Sugars were applied to papers as a number of superimposed spots from 1 % aqueous solutions. Furfurals were spotted from 5 % solutions in methanol and dried at room temperature. Papers were developed by downward irrigation, in ethyl acetate–pyridine–water (2:1:2)[6] and in butanol-1–ethanol–water (40:11:19)[7]. Uronic acids were separated by development in ethyl acetate–acetic acid–formic acid–water (9:1.5:0.5:2)[8]. Although an acetone solution of the spray reagents was devised for dipping papers it was found that more consistent results were obtained when the papers were lightly sprayed with this solution. The sprayed, dried papers were finally heated in a fan-stirred thermostatic oven and the temperature checked on a thermometer hung next to the paper. A variation in temperature of not more than ± 2° about the specified temperature was generally obtained and considered satisfactory.

Spray reagents

(*a*) *Mixed diphenylamine–aniline reagents.* The normal reagent contained diphenylamine (4 g), aniline (4 ml) and syrupy (80 %) orthophosphoric acid (20 ml) in acetone (200 ml). Modified versions of this spray in which the aniline concentration was changed to 6, 2 or 1 ml were also prepared.

(*b*) *Split reagent.* Two separate solutions were prepared containing (1) diphenylamine (4 g), phosphoric acid (20 ml) and acetone (200 ml) and (2) aniline (4 ml), phosphoric acid (20 ml) and acetone (200 ml). In using these solutions the paper was sprayed with either one of them, dried, heated, then sprayed with the second solution and again dried and heated.

(*c*) *Triphenylamine reagent.* Both the normal mixed and split reagents were prepared except that the diphenylamine was replaced with triphenylamine.

All of the sprays were made as required and used fresh. Provided they were stored at 0°, however, the sprays appeared to keep satisfactorily for 2–3 weeks.

RESULTS

Effects of various conditions on colour development

The effect of time and temperature of heating and of sugar concentration were investigated in preliminary experiments in which the sugars were spotted on circles of filter paper and tested without solvent development.

(*a*) *Concentration of sugar.* Each sugar was tested at three concentrations which were approximately 10–15 μg (2 spots), 25–30 μg (5 spots) and 50–60 μg (10 spots), respectively. Increasing the concentration did not affect the shade of colour but merely increased its intensity. The sugar concentration of 50–60 μg was used on the final test chromatograms, as spots of reasonably strong intensity were desired.

(*b*) *Temperature.* Sprayed papers were heated at 50, 60, 70, 80, 90 and 100°. The sugars could be divided into two groups according to their optimum temperature for colour development and differentiation. These were (1) uronic acids, aldoheptoses, aldohexoses, ketohexoses, methyl pentoses and disaccharides containing one of these sugars as the reducing unit, optimum temperature 80°; and (2) pentoses and pentose disaccharides, optimum temperature 60°.

(*c*) *Time of heating.* At the appropriate temperature, colour appeared after 2 min and was well developed at the end of 4 min. Longer heating did not increase or improve colour intensity. At temperatures above optimum the spots were more intense and quicker in developing; however, in many cases only brown-black colours were obtained. Thus at 80° the pentose colour was transient and rapidly changed to a dirty brown. At 60° group (1) compounds produced no colour after 5 min heating; they do not, therefore, interfere with the identification of pentoses at this temperature.

TABLE I

COLOUR REACTIONS ON PAPER CHROMATOGRAMS OF SUGARS AND DIPHENYLAMINE–ANILINE REAGENT

Sugars	*Colour*
(*1*) *Papers heated at 80° for 4 min*	
L-Rhamnose, L-fucose	orange
D-Glucoheptose (L. Light & Co.), D-glycero-L-galaheptose, D-glycero-D-guloheptose	bright purple-blue
D-Galacturonic, β-1,4-digalacturonic, D-glucuronic, D-mannuronic acids, glucuronolactone, mannuronolactone	bright red-brown
Maltose, cellobiose, lactose	bright blue
Leucrose	pale blue-green
L-Gulose, D-galactose, D-glucose, D-mannose	grey-brown
Isomaltose, gentiobiose, melibiose, laminaribiose	grey-brown or yellow-brown
Sophorose	yellow-orange
D-Fructose, L-sorbose, planteobiose, inulobiose	yellow or yellow-brown
Turanose	purple-brown
D-Glucosamine, D-galactosamine	nil.
(*2*) *Papers heated at 60° for 4 min*	
D-Xylose, L-arabinose, D-ribose, D-lyxose	bright greenish-blue
Xylobiose (4-O-β-D-xylopyranosyl-D-xylopyranose)	yellowish-green
Rhodymenabiose	green

Colours produced by sugars with the mixed reagents

Paper chromatograms of the sugars were developed, sprayed and heated according to the conditions defined above. The results obtained with the normal mixed reagent are listed in Table I.

After heating, the papers were washed by dipping in water. Whilst the colours of group (1) sugars were either unaffected or slowly washed from the paper, the green pentose (group 2) colours changed rapidly to bright red-browns. This latter colour-change could be reversed by treatment with strong acid.

In the normal mixed reagent the molecular ratio of diphenylamine: aniline is approximately 1:2. A representative selection of sugars, on chromatograms, (D-glucose, maltose, α-D-glucoheptose, D-fructose, L-fucose, isomaltose, D-xylose and L-arabinose) were sprayed with reagents in which the ratios were approximately 1:3, 1:1 and 1:0.5 (aniline 6, 2 and 1 ml/200 ml of acetone, respectively). With group (1) sugars the same results as those listed in Table I were obtained at 80° with the 1:3 spray. The other two sprays gave weaker colours which were often slower in developing and, in the case of reducing aldohexoses, bluer in colour (*cf.* Table II). With the pentoses, however, the 1:3 spray at 60° gave much more intense greenish-blue colours which developed quickly (maximum intensity in 2 min). Similar results were obtained with the two pentose disaccharides. This modified spray is recommended as preferable for identifying pentoses. The 1:1 and 1:0.5 sprays gave with the pentoses yellow-green and weak brown colours, respectively.

TABLE II

COLOUR REACTIONS ON PAPER CHROMATOGRAMS OF SUGARS SPRAYED WITH TWO SUCCESSIVE REAGENTS

Sugars	*Diphenylamine then aniline*		*Aniline then diphenylamine*	
(1) Papers heated at 80° for 5 min after each spraying				
L-Rhamnose, L-fucose	nil	yellow	orange	orange
Heptoses of Table I	faint yellow	purple-blue	yellow-brown	brown-blue
Uronic acids of Table I	light red-brown	purple-brown	brown	brown
Maltose, cellobiose, lactose	nil	bright-blue	faint brown	bright blue
Leucrose	nil	pale blue-green	nil	pale blue-green
L-Gulose, D-galactose, D-glucose, D-mannose	nil	greenish-blue	yellow-brown	yellow-brown
Isomaltose, gentiobiose, laminaribiose, melibiose, sophorose	nil	greenish-blue	brown	brown
D-Fructose	light brown	bright red-brown	yellow	yellow
L-Sorbose	pale yellow	yellow	yellow	yellow
Planteobiose, inulobiose	pale yellow	yellow-brown	yellow	yellow
Turanose	light purple	bright magenta	brown	purplish-brown
(2) Papers heated at 60° for 5 min after each spraying				
D-Xylose, L-arabinose, D-lyxose, D-ribose	nil	weak red-brown	yellow-brown	greenish-yellow

Colours produced by sugars with the split reagent

Chromatograms were sprayed, as described above, with both combinations of the two reagents. After each spraying, the papers were heated at the appropriate temperature for 5 min. The results obtained are listed in Table II.

Colours produced by sugars with triphenylamine

A representative selection of sugars (D-fructose, L-sorbose, D-glucose, α-D-glucoheptose, L-rhamnose, D-galacturonic acid, maltose, melibiose, turanose and L-arabinose) was treated with mixed triphenylamine–aniline reagent and with the split reagents. With mixed reagent and with aniline alone all of the sugars gave yellow to brown colours. The colours on the aniline-treated paper remained unchanged after reaction with triphenylamine. With triphenylamine alone fructose, sorbose and turanose gave dull bluish-purple colours and all of the other sugars failed completely to react. On subsequent treatment with aniline the ketose colours changed rapidly to yellow and the other sugars developed weak brown colours, except maltose which still failed to give any colour.

Colours produced by furfurals with the spray reagents

Purified furfural was tested with mixed and "split" reagents immediately after spotting on filter paper discs. 5-Hydroxymethylfurfural was tested in this way and after running on chromatograms. With all treatments of diphenylamine and aniline furfural gave a yellow colour at room temperature which turned orange on heating at 60°, and 5-hydroxymethylfurfural gave a bright yellow colour which remained unchanged on heating at 60 or 80°. With the triphenylamine reagent alone both furfural and 5-hydroxymethylfurfural gave no colour in the cold, but a bluish-purple colour on heating for a few min at 80°. Subsequent treatment with aniline rapidly changed this colour to bright yellow. With mixed reagent both compounds gave bright yellow colours.

DISCUSSION

Summary of specific colour reactions given by the reagents

Inspection of Tables I and II indicates that the following specific colour reactions given by sugars with diphenylamine–aniline reagents may be of value for identification purposes.

(a) With mixed reagent (diphenylamine–aniline, 1:2) heated at 80°. Aldoheptoses, bright purple-blue. Uronic acids, bright red-brown. Methyl pentoses, orange. 1,4-linked reducing aldohexose oligosaccharides, bright blue. In leucrose (5-O-α-D-glucopyranosyl-D-fructopyranose[9]) the glycosidic link at position 5 of fructopyranose stands in the same relation to the reducing group as does the glycosidic linkage in a 1,4-linked aldohexose disaccharide. The blue-green colour given by this sugar (compared with other ketose disaccharides) is, therefore, to be expected if the rule of SCHWIMMER AND BEVENUE[2] holds in this modified form for all hexose disaccharides.

(b) With mixed reagents (diphenylamine–aniline, 1:2 or preferably 1:3) heated at 60°. Pentoses and reducing pentose oligosaccharides give bright greens or greenish-blues, which change to bright red-brown on washing with water.

(c) With diphenylamine (heated at 80°) followed by aniline (heated at 80°). Aldoheptoses, and 1,4-linked reducing aldohexose oligosaccharides give the same specific colours as with the mixed reagent. Disaccharides with a reducing aldohexose unit (other than 1,4-linked) and aldohexoses, greenish-blue. Turanose, bright magenta. Uronic acids, purple-brown. Fructose (bright red-brown) is clearly distinguished from sorbose (yellow).

All of the colour reactions listed above are reproducible and distinctive. There may be some danger, however, unless appropriate standards are run, of confusing the aldoheptose blue colour with the blue produced by 1,4-linked aldohexose disaccharides. This is important as the normal mixed reagent has been accepted as a means of demonstrating the presence of the 1,4-link in a disaccharide. In a total hydrolysate of a polysaccharide the danger of such confusion would not arise and in this case diphenylamine–aniline provides a useful confirmatory test for aldoheptoses.

Less clear-cut colour reactions are obtained with other sugars and these may be of some value for identification purposes. Although the present results were obtained from chromatograms carrying 50–60 μg of sugar, the reagents are more sensitive. With many sugars the specific colour reaction could be obtained with as little as 10–15 μg. For good colour reactions, however, sugar concentrations of about 30–50 μg are to be preferred.

Nature of the reactions involved

Under the conditions used glycosidic linkages, other than exceptionally acid-labile ones, are apparently not hydrolysed. Thus α,α-trehalose gives no colour, whilst sucrose gives the colour expected for a mixture of glucose and fructose. Many, but not all, of the colours given by the normal mixed reagent (Table I) appear to be the sum of the colours expected if a major portion of the sugar first reacts with aniline followed by diphenylamine, and the rest reacts first with the diphenylamine. Increasing the aniline content, as would be expected, gives the same types of colours; whilst decreasing it, at least with aldohexoses, gives the type of colour (blue) produced by diphenylamine followed by aniline (Table II). The pentose colours do not appear to be the sum of the colour products of the two separate reactions although composition of the spray is important.

Many of the colour reactions of sugars with acidic reagents are ascribed to the preliminary conversion of the sugar, by acid, to furfural or a derivative which then reacts with the reagent to give a coloured compound[10]. Furfural (from pentoses) and 5-hydroxymethylfurfural (from hexoses) give the same colours (orange and yellow respectively) with all of the diphenylamine and aniline treatments used. With triphenylamine both give the same bluish-purple colour. Although many of the sugars do give a furfural type of colour with aniline, ketoses are the only sugars which tend to give the predicted furfural type of colour with all of the reagents, including triphenylamine. Ketohexoses undergo conversion to 5-hydroxymethylfurfural much more readily than aldohexoses[10]. The colours given by pentoses with mixed diphenylamine–aniline and by aldohexoses with diphenylamine followed by aniline would not be expected if furfural formation was a prerequisite. The failure of the pentoses to react with triphenylamine and of the aldohexoses to react with di- or tri-phenylamine alone is also contrary to any theory based on furfural production. In these cases at least some other reaction must be involved.

Most simple sugars form N-glycosylamines with primary or secondary amines[11]. Under acid conditions these compounds readily undergo an Amadori rearrangement to give either an N-substituted 1-amino-1-deoxy-2-ketose or its 1-2 enolic form[12]. According to GOTTSCHALK[13] these compounds readily give rise to a furfural and amine on further acid treatment. The above sequence of reactions may be involved in the formation of colours when many of the sugars react with aniline alone. The glycosylamines formed with diphenylamine may not undergo this transformation, hence no colour is produced. On subsequent treatment with aniline the diphenyl N-glycosylamine may modify the aniline reaction to give rise to a different colour (Table II). Presumably triphenylamine cannot form a glycosylamine; no reaction normally occurs, except with ketoses which easily form a furfural, and there is little or no interference in the subsequent aniline reaction.

It is not possible to suggest a mechanism for colour formation in the case of the more complex sugars such as the uronic acids, heptoses and 1,4-linked aldohexose disaccharides. Diphenylamine can be involved in reactions with sugars other than the ones suggested above. Thus DISCHE[14] and GIBBONS, MORGAN AND GIBBONS[15] find that aldohexoses and ketohexoses give a bright blue colour when heated at 100° for 10 min with acid diphenylamine. These results are quite different from those reported in the present study with diphenylamine alone (Table II).

It is evident that the reactions between sugars and amines to produce coloured compounds are extremely complex. Nevertheless, in the case of diphenylamine and aniline the colours which can be obtained with both the mixed and split reagents should be of value in identifying sugars on chromatograms.

ACKNOWLEDGEMENTS

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SUMMARY

Conditions have been defined in which mixed acidic diphenylamine–aniline sprays give, on paper chromatograms, specific colour reactions with uronic acids, aldoheptoses, methyl pentoses and pentoses.

When the reagents are applied as two successive sprays, *i.e.* diphenylamine followed by aniline, additional specific colour reactions are obtained with (1) all aldohexoses and reducing aldohexose disaccharides (other than those containing a 1,4-link) and (2) with some ketoses. This split reagent is able to distinguish clearly between L-sorbose and D-fructose.

The possible reactions involved in the formation of the colours are discussed.

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GAS–LIQUID CHROMATOGRAPHY OF ALLYLIC ALCOHOLS AND RELATED BRANCHED-CHAIN ACIDS

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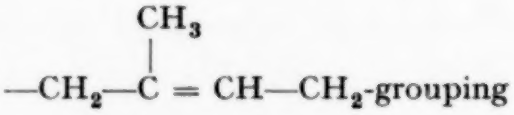
During studies on the biosynthesis of squalene and cholesterol from [2-^{14}C] mevalonic acid (3,5-dihydroxy-3-methylpentanoic acid) in liver enzyme preparations a number of volatile neutral and acidic products have been encountered[1,2] which in gas–liquid chromatograms showed retention volumes different from those of any hitherto recognized animal product. The origin of these from mevalonate suggested that they might be isoprenoid substances, *i.e.*, containing the characteristic



$$-\mathrm{CH_2}-\overset{\mathrm{CH_3}}{\overset{|}{\mathrm{C}}}=\mathrm{CH}-\mathrm{CH_2}\text{-grouping}$$

Consequently it became necessary to establish the behaviour in gas–liquid chromatograms of both alcohols and acids belonging to this series. In order to simplify nomenclature of substances analysed it is proposed to refer to the alcohols and the acids, which can be clearly dissected into isoprenoid units, as prenols (or polyprenols) and prenoic (or polyprenoic) acids, and collectively as polyprenoid substances. Thus 3,3-dimethylallyl alcohol may be called monoprenol, and farnesol *trans-trans*-triprenol; *trans*-geranic acid becomes *trans*-diprenoic acid and the all-*trans*-3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraenoic acid all-*trans*-tetraprenoic acid respectively. Similar nomenclature may be applied to the tertiary alcohols linalool and nerolidol, *i.e.*, *tert.*-diprenol and *tert.*-triprenol.

In this paper the relative retention volumes of prenols (primary and tertiary) and of the methyl esters of prenoic acids on columns with polar and non-polar stationary phases are being reported, together with values obtained for the methyl esters of *n*-fatty acids under identical conditions.

EXPERIMENTAL

Gas–liquid chromatography

The apparatus was essentially identical with that described by MARTIN AND JAMES[3] in which a gas-density meter is employed as a detection device. All measurements were made at one temperature, at the boiling point of ethylene glycol (197°). The gas-density meter, in which the original principles of MARTIN AND JAMES[3] were retained, was of a new design and will be described in detail elsewhere.

The chromatographic columns were 120 cm long and of 4 mm internal diameter; the support for the stationary phases was Celite, 120–150 mesh size, and washed with acid and alkali. The stationary phases, dissolved in solvents, were mixed with the support in the ratio of 15:100, or 20:100 (w/w) the solvents being slowly evaporated off.

Since the introduction of polyester stationary phases in gas–liquid chromatography[4,5] several varieties of these have been described. We used an ethylene glycol–adipic acid polyester made according to JAMES[6]. The polyester, without purification, was added to the supporting Celite as a solution in acetone. The "adipate" columns gave very good separations of both the prenols and methyl prenoates, but it was essential to condition the columns first for at least 1 week at 197° while a continuous stream of N_2 was being passed through them. Without such conditioning, prenols with retention volumes higher than that of nerolidol were retained completely by the columns, probably on account of esterification with free carboxyl groups on the adipate polymer. This was the case even though an excess of ethylene glycol was used during the preparation of the polymer. The efficiency of adipate columns was usually between 1800 and 2700 theoretical plates measured with methyl stearate.

Apiezon-L vacuum grease was used for columns with a non-polar stationary phase; it was mixed with the Celite support in a solution of light petroleum (b.p. 40–60°). These columns could be used satisfactorily after a few hours of conditioning; their theoretical plate efficiency varied between 3200 and 3700.

Compounds analysed and their sources

The compounds analysed are listed in Table I. Most of the prenols and prenoic acids were the generous gifts of colleagues or manufacturing organizations, whose help we gratefully acknowledge.

3,3-Dimethylallyl alcohol was provided by Dr. J. W. CORNFORTH, of the National Institute for Medical Research, London; it was a pure specimen.

Geraniol and its *cis*-isomer, *nerol*, methyl *trans*-geranate and methyl *cis*-geranate were the gifts of Dr. B. C. L. WEEDON of the Imperial College of Science and Technology. Each of these preparations was essentially pure.

Linalool (pure) was the preparation of A. BOAKE, Roberts and Co., London; the same firm's product "Meranol", which is pure geraniol, was also used.

Specimens of *nerolidol* and of *farnesol* were the gifts of Dr. M. STOLL of Firmenich et Cie, Geneva, Switzerland. The farnesol—we were informed—was made by the allylic rearrangement of nerolidol and consequently it contained both the *trans-trans* and *cis-trans* isomers of farnesol and about 11% of unchanged nerolidol which was identical with the major component of the specimen of nerolidol derived from natural sources.

Dihydronerolidol (3,7,11-trimethyldodeca-6,10-dien-3-ol) was synthesized by the

Grignard reaction from *trans*-geranylacetone and ethyl magnesium bromide[7]. Gas–liquid chromatography showed it to contain one component only.

Ethyl farnesoate (ethyl 3,7,11-trimethyldodeca-2,6,10-trienoate). This was synthesized by the reaction of ethoxyacetylene and geranylacetone.

Ethoxyacetylene (5.9 g) in ether (30 ml) was added during 30 min to ethyl magnesium bromide (from 1.6 g magnesium) in ether (30 ml). When evolution of ethane had ceased *trans*-geranylacetone (10.9 g) in ether (20 ml) was added during 30 min; slight warming occurred and the ethoxyethenyl magnesium bromide dissolved. After 4 h at room temperature saturated ammonium chloride (80 ml) was added and the product isolated by ether extraction. Evaporation of the dried solution at low pressure left 14.7 g of crude product, 7.5 g of which was dissolved in tetrahydrofuran (40 ml) and 2 N H_2SO_4 (1.8 ml) added with swirling and cooling. After 30 min saturated $NaHCO_3$ solution (10 ml) was added, the tetrahydrofuran evaporated at low pressure, and the product isolated with ether. Ethyl farnesoate (6.6 g), b.p. 106°/0.005 mm, was obtained. (Found: C, 77.1; H, 10.3. Calc. for $C_{17}H_{28}O_2$, C, 77.2; H, 10.6%.)

It followed from the mode of synthesis that the product should contain the *trans-trans* and *cis-trans* isomers of ethyl farnesoate. Gas–liquid chromatography both on polar and nonpolar columns revealed two components only and in equal amounts as expected; the component with the larger retention volume was assumed to be the *trans-trans* isomer, an assumption justified by the fact that both geraniol and methyl *trans*-geranate have retention volumes higher than their respective *cis* isomers.

The identity of the ethyl farnesoate preparation was further established from its reduction to farnesol. Ethyl farnesoate (1.3 g) in ether (5 ml) was added dropwise to a solution of $LiAlH_4$ (0.4 g) in ether (12 ml) at — 30°, the mixture being stirred for 1.5 h when water (2 ml) was added slowly and the reaction mixture worked up in the normal way. Farnesol (0.82 g), b.p. 90–91/0.001 mm, n_D^{24} 1.4860, was obtained as colourless oil. (Found: C, 80.8; H, 11.8. Calc. for $C_{15}H_{26}O$, C, 81.1; H, 11.7%.) Gas–liquid chromatography of this preparation showed two components only identical with the *cis-trans* and *trans-trans*-farnesol obtained from rearrangement of nerolidol.

The synthesis of ethyl farnesoate by the method described is mentioned by CALIEZI AND SCHINZ[8], but is not described in the experimental section of their paper.

Methyl farnesoate. Free farnesoic acid was obtained by the hydrolysis (0.5 N NaOH in 50% ethanol) for 4 days at room temperature of ethyl farnesoate followed by the usual extraction of the free acid. Saponification of ethyl farnesoate with 1 N methanolic KOH by refluxing is unsatisfactory as it produces at least three artefacts (unidentified) at the expense of the *cis-trans* isomer. Even the hydrolysis at room temperature produced an additional component at the expense of the *cis-trans* isomer, this is believed to be the 3,7,11-trimethyldodeca-3,6,10-trienoic acid.

Methyl farnesoate was prepared by treating farnesoic acid dissolved in ether with a dilute solution of diazomethane in ether at 0°. Methylation with diazomethane is entirely satisfactory and introduces no artefacts provided only a small excess of diazomethane is used; this has been checked by preparing the methyl esters also from silver salts with methyl iodide. The methyl farnesoate preparation was identical chromatographically with specimens synthesized by three different methods.

3,7,11-Trimethyldodecanoic acid. This saturated analogue of farnesoic acid was made by catalytic hydrogenation of ethyl farnesoate. After hydrogenation the free acid and methyl ester was prepared in the usual way. Gas–liquid chromatography showed only one component, which was identical with another specimen of 3,7,11-trimethyldodecanoate given to us by Dr. KARL FOLKERS of the Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey, U.S.A.

Geranyl-linalool (3,7,11,15-tetramethylhexadeca-1,6,10,14-tetraen-3-ol = *tert.*-tetraprenol). This C_{20} homologue of nerolidol was the gift of Dr. KARL FOLKERS; it contained two components assumed to be the *trans-trans* (6:10) and *cis-trans* (6:10) isomers. In experiments with liver enzyme preparations two neutral substances synthesized from [2-^{14}C]mevalonate were found with retention volumes identical with those of the two isomers of the *tert.*-tetraprenol.

In Table I and in Figs. 1 and 2 data are given also for the C_{20} all-*trans*-tetraprenol (geranylgeraniol); these have been obtained by extrapolation. In biosynthetic experiments with mevalonate a neutral substance was found which had a retention volume identical with the value obtained by such extrapolation.

3,7,11,15-Tetramethylhexadeca-2,6,10,14-tetraenoic acid (tetraprenoic acid). A specimen containing four geometrical isomers of the tetraprenoic acid was provided also by Dr. KARL FOLKERS. Two of the major components of this specimen (after methylation) had retention volumes very nearly identical with those predicted by extrapolation for the all-*trans* and *cis-trans* isomers.

RESULTS AND DISCUSSION

All data obtained are contained in Tables I and II, and in Figs. 1 and 2. The retention volume (R_v) of *trans-trans*-farnesol was taken as the reference, retention volumes having been measured from the beginning of the air peaks to the maxima of each fraction. The R_v values of the methyl esters of representative *n*-fatty acids, relative to that of farnesol, are included in order that our data may be fitted to those of others.

It will be seen from Table I that on both types of columns a few substances overlapped. Two substances differing in their retention volume by 2 % or less could not be distinguished on our records, but a difference of 4 % caused a noticeable skewing of the peaks when two such substances were run together. At 8 % difference—although the fractions would overlap—two substances could be distinguished readily. In order to obtain the best estimate of the R_v in such instances, the overlapping substances were chromatographed repeatedly, one by one, with several others whose R_v values relative to farnesol have already been determined. All data in Table I are the mean of 4–6 determinations and are reproducible under our conditions to within $\pm$ 2 %.

On the adipate columns the following pairs of substances could not be separated: nerol–methyl dodecanoate, nerolidol–dihydronerolidol, nerolidol–methyl myristoleate, and methyl *trans-trans*-farnesoate–methyl palmitoleate. On the apiezon columns the following pairs did not separate: methyl *trans*-geranate–methyl *n*-decanoate, and *trans-trans*-farnesol–methyl *cis-trans*-farnesoate. However, as there were no two identical overlapping pairs of substances on the two columns, it is clear that all the substances studied may be identified with reasonable confidence by the use of polar and non-polar stationary phases in gas–liquid chromatography.

TABLE I

RETENTION VOLUMES OF POLYPRENOID SUBSTANCES AND OF *n*-FATTY ACIDS RELATIVE TO THAT OF *trans-trans*-FARNESOL ON COLUMNS WITH ETHYLENE GLYCOL–ADIPIC ACID POLYESTER AND APIEZON-L STATIONARY PHASES AT 197°

Substance	R_v *(adipate)*	R_v *(apiezon)*
Prenols, primary		
3,3-Dimethylallyl alcohol	0.037	0.0185
Nerol (*cis*-geraniol)	0.172	0.124
Geraniol	0.193	0.136
cis-trans-Farnesol	0.893	0.912
trans-trans-Farnesol	1.000	1.000
all-*trans*-Tetraprenol (C_{20})	4.800*	7.350*
Prenols, tertiary		
Linalool	0.074	0.0704
Nerolidol	0.385	0.515
Dihydronerolidol (3,7,11-trimethyldodeca-6,10-dien-3-ol)	0.385	0.618
tert.-Tetraprenol (C_{20}): *cis-trans*	1.560	3.14
(geranyl-linalool) *trans-trans*	1.850	3.790
Prenoic acids, as methyl esters		
cis-Geranate	0.116	0.143
trans-Geranate	0.147	0.175
3,7,11-Trimethyldodecanoate	0.230	0.720
cis-trans-Farnesoate	0.580	0.990
trans-trans-Farnesoate	0.736	1.210
cis-trans-trans-Tetraprenoate (C_{20})	2.910	6.910
all-*trans*-Tetraprenoate (3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenoate)	3.520	8.100
n-Fatty acids, as methyl esters		
Decanoate	0.093	0.174
Dodecanoate	0.178	0.414
Tetradecanoate	0.339	0.990
Hexadecanoate	0.646	2.410
Octadecanoate	1.230	5.800

* Values obtained by extrapolation, see also text.

The behaviour of the adipate columns is remarkable in that when the logarithm of the R_v values for substances within one homologous series is plotted against the number of carbon atoms (Fig. 1), two sets of parallel lines are obtained: one set relates to the prenols and the second set to all the methyl esters of the acids. These two sets of lines are very nearly parallel. Similar plot of data for apiezon columns

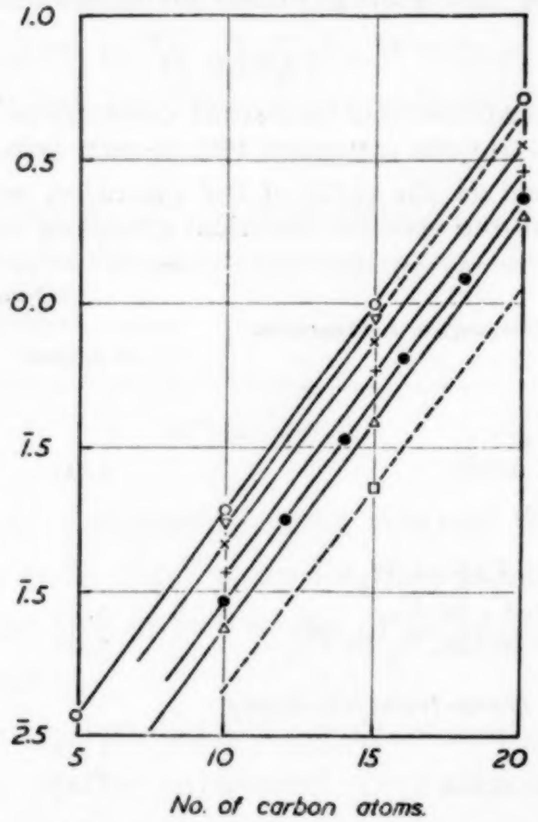


Fig. 1. Relationship between $\log_{10}$ relative retention volume on an adipate column and the number of carbon atoms for homologous series. ○ = Primary *trans*-prenols. ▽ = Primary *cis-trans*-prenols. × = *trans*-Prenoates (Me esters). + = *cis-trans*-Prenoates (Me esters). ● = *n*-Fatty acids (Me esters). △ = *tert*.-Prenols. □ = 3,7,11-Trimethyldodecanoate (Me ester).

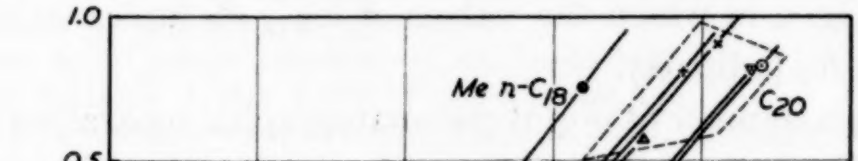


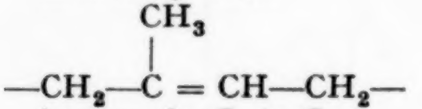
Fig. 2. Relationship between $\log_{10}$ relative retention volumes on apiezon and on adipate.

showed again a set of parallel lines for the prenols, two parallel lines for the methyl esters of the *trans* (or all-*trans*) and *cis* (or *cis-trans*) prenoic acids. The slope of the line giving the relationship for the methyl esters of *n*-fatty acids was, however, very different from the slope of the polyprenoid substances. This is reflected also in the slope of lines on Fig. 2 in which the values of $\log_{10} R_v$ (apiezon) are plotted against the values of $\log_{10} R_v$ (adipate).

TABLE II

SEPARATION FACTORS FOR CHARACTERISTIC CHEMICAL GROUPINGS IN GAS–LIQUID CHROMATOGRAPHY AT 197° ON ETHYLENE GLYCOL–ADIPATE POLYESTER AND APIEZON-L COLUMNS

The separation factor is defined as the ratio of the retention volume of two substances differing in respect of one specific chemical grouping or configuration.

Chemical grouping, or configuration	*Separation factors*	
	on adipate	*on apiezon-L*
—CH_2—CH_2— in *n*-fatty acids	1.90	2.41*
 $-CH_2-\overset{\displaystyle CH_3}{\overset{|}{C}}=CH-CH_2-$		
in prenols, C_5 to C_{15}	5.20	7.35
in prenoic acids, C_5 to C_{15}	5.02	6.91
trans/cis, or *trans-trans/cis-trans*		
in prenols	1.12	1.10
in prenoic acids	1.27	1.22
primary OH/tertiary OH	2.60	1.94

* This is a value very close to that expected (2.43) from the separation factor for the —CH_2- group of 1.56 given by JAMES[6].

From the data a number of useful chromatographic separation factors, characteristic for certain chemical groupings or configurations, could be derived; these are shown in Table II. It is of interest to note, however, that the separation factor for a difference of one isoprenoid unit between nerolidol (C_{15}) and the *tert.*-tetraprenol (C_{20}) or between methyl farnesoate and methyl all-*trans*-tetraprenoate (C_{20}) was found to be only 4.80 on the adipate columns, although this value was 5.20 for substances in the C_5 to C_{15} range.

There was one finding, which calls for caution in assigning chemical structures for unknown substances on the basis of gas–liquid chromatographic analysis made on one type of column alone. Although it is true that on apiezon columns the esters of saturated *n*-fatty acids have larger retention volumes than the corresponding unsaturated ones, this relationship in the polyprenoic acid series is, apparently, reversed: the R_v (apiezon) values for methyl *trans-trans*-farnesoate and methyl *cis-trans*-farnesoate were 1.21 and 0.99 respectively, and for the corresponding saturated analogue, methyl 3,7,11-trimethyldodecanoate, 0.72, a value only little higher than that expected for methyl *n*-tridecanoate. On the other hand, dihydronerolidol (3,7,11-trimethyldodeca-6,10-dien-3-ol) had a relative R_v (apiezon) of 0.618 against 0.515 for nerolidol, the two substances being inseparable on adipate columns.

ACKNOWLEDGEMENTS

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SUMMARY

Relative retention volumes of polyprenols and related branched-chain acids have been determined on columns with a polar and a non-polar stationary phase in gas-liquid chromatography. By the use of two types of columns these substances may be identified with good confidence.

The synthesis of ethyl farnesoate (ethyl 3,7,11-trimethyldodeca-2,6,10-trienoate) and the preparation from it of the saturated 3,7,11-trimethyldodecanoate and of farnesol are also described.

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ÜBER DIE ADSORPTION VON POLONIUM AN ZELLULOSE UND GLAS

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VORBEMERKUNG

Im Zusammenhang mit neueren Untersuchungen M. LEDERERS über das Verhalten des Poloniums in der Papierchromatographie scheint es von Interesse, einige Daten über die Adsorption dieses Elements mitzuteilen, die in einer im Jahr 1950 abgeschlossenen Dissertation enthalten sind[1]. Die Versuche wurden am I. Chemischen Institut der Universität Wien (jetzt Anorganisch- und physikalisch-chemisches Institut) durchgeführt.

Die Adsorption des Poloniums an Glas war schon früher von LENG[2], HAISSINSKY[3] und STARIK[4] untersucht worden (Andere Untersuchungen über diese Frage sind vorläufig nicht allgemein zugänglich[4a].) In der vorliegenden Arbeit wurde die Adsorption des Poloniums an Glas und Zellulose untersucht, wobei der Einfluss der H^+-Ionenkonzentration und von Elektrolytzusätzen geprüft wurde. Die Versuche wurden durchgeführt, um einen besseren Einblick in die Gesetzmässigkeiten der Radiokolloidadsorption zu erhalten.

EXPERIMENTELLES

Die verwendeten Poloniumlösungen enthielten $3 \cdot 10^{-11}$ bis $3 \cdot 10^{-10}$ Mol Polonium pro l. Zur Bestimmung des Poloniums wurden gewogene Mengen der Lösung auf Silberblech eintrocknen gelassen und die Intensität der α-Strahlung mit einem Blättchenelektroskop bestimmt; diese Messungen waren innerhalb von 2 % reproduzierbar. Um Aussagen über den absoluten Gehalt an Polonium machen zu können, wurde eines dieser Blättchen im Wiener Institut für Radiumforschung mit einem dort aufbewahrten Standard verglichen.

Die verwendeten Glassorten waren Glaswolle aus weichem Natronglas und Glaspulver aus österreichischem Wirag-Glas (ähnlich dem Jenaer Glas). Bei der Bestimmung der Poloniumadsorption an Glas wurde die Aktivität der Lösung vor und nach der Adsorption gemessen. Nur bei den Versuchen mit Zusätzen von Elektrolyten wurde die Aktivität des vom Glas eluierten Poloniums gemessen. So wurde vermieden, dass die beim Verdunsten der Lösung sich bildende, verhältnismässig dicke Salzschichte die α-Strahlen zu stark absorbiert und so die Resultate verfälscht.

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[*] Jetzt in der Firma Loba-Chemie, Wien, XIX.

Bei den Versuchen mit Zellulose wurde zumeist sowohl die Aktivität der Lösung als auch die Aktivität des von der Zellulose eluierten Poloniums gemessen. Die Summe dieser beiden Messungen wurde als 100 % betrachtet, wodurch der Einfluss der ebenfalls Polonium adsorbierenden Wand des Glasgefässes ausgeschaltet wurde. Bei Versuchen mit Salzzusätzen wurde aber auch hier nur die Aktivität des aus dem Adsorbens eluierten Poloniums gemessen.

Die Einstellung der H^+-Ionenkonzentration erfolgte durch Zugabe von NaOH-Lösung zu der Poloniumstammlösung, die ursprünglich 0.1 *M* an HCl war. Daher gilt für die Poloniumlösungen, aus denen die Adsorption erfolgte: $[Cl^-] = [Na^+] + [H^+] = 0.1$.

Bei allen Versuchen betrug die Adsorptionsdauer eine Stunde; Vorversuche hatten nämlich gezeigt, dass bei Adsorptionszeiten von 30 Minuten und von 3 Stunden die Poloniumadsorption gleich gross war. Bei jedem Versuch wurden 10 ml Lösung eingesetzt. Bei den Versuchen mit Zellulose verwendeten wir 0.1 g des Adsorbens, bei den Versuchen mit Glas wurden 0.2 g Glaswolle bzw. 1 g Glaspulver (beides entspricht etwa 200 cm² Oberfläche) verwendet.

ERGEBNISSE

Die wesentlichen Resultate über die Adsorption von Polonium an Zellulose sind in Fig. 1 dargestellt. Kurve I zeigt die Abhängigkeit der Adsorption vom pH-Wert bei einer Poloniumkonzentration von $3 \cdot 10^{-11}$ Mol/l, Kurve II die pH-Abhängigkeit bei einer Konzentration von $3 \cdot 10^{-10}$ Mol/l.

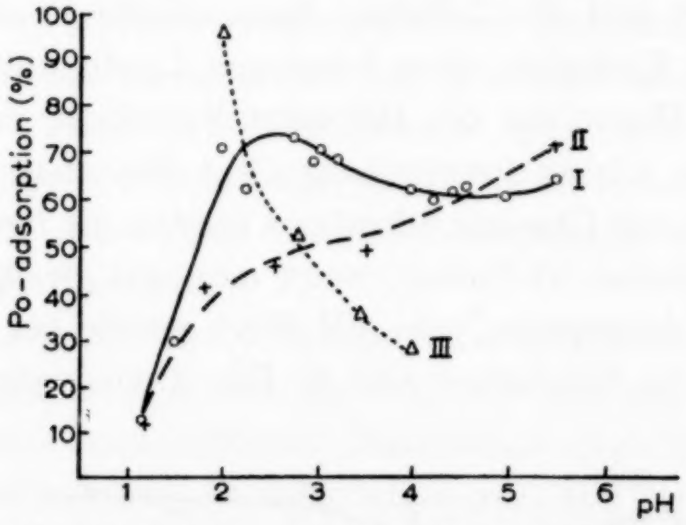

Fig. 1. pH-Abhängigkeit der Adsorption von Polonium an Zellulose. I: $[Po] = 3 \cdot 10^{-11}$. II: $[Po] = 3 \cdot 10^{-10}$. III: $[Po] = 2.5 \cdot 10^{-11}$; $[La(NO_3)_3] = 0.03$. Für alle Versuche: $[Na^+] + [H^+] = [Cl^-] = 0.1$.

Die Beeinflussung der Adsorption durch Elektrolytzusätze (Barium- und Lanthannitrat) wurde bei verschiedenen pH-Werten untersucht. Zugaben von Bariumnitrat bis 0.1 Mol/l wurden bei pH 3.2 und 4.2 bei Poloniumkonzentrationen von $3 \cdot 10^{-11}$ und $1.5 \cdot 10^{-10}$ Mol/l vorgenommen. Dabei wurden keine Änderungen der Adsorptionswerte gegenüber den Werten gefunden, die ohne diese Zusätze auftreten. Merkliche Veränderungen ergaben sich jedoch bei Zusätzen von Lanthannitrat

(Tabelle I) zu einer Poloniumlösung von $2.5 \cdot 10^{-11}$ Mol/l. Es scheint bemerkenswert, dass bei einem niederen pH-Wert (pH = 2.0) eine Steigerung der Adsorption, bei höheren pH-Werten (pH $\geqslant$ 2.8) eine Verringerung auftritt. Während bei den höheren pH-Werten schon sehr kleine Zusätze eine starke Wirkung ausüben, wird bei pH 2.0 eine beträchtliche Adsorptionssteigerung erst durch verhältnismässig grosse Zusätze herbeigeführt. Die bei Zusatz von 0.03 Mol Lanthannitrat pro l erhaltenen Adsorptionswerte sind auch in der Fig. 1 wiedergegeben (Kurve III).

TABELLE I

BEEINFLUSSUNG DER ADSORPTION VON POLONIUM AN ZELLULOSE DURCH LANTHANNITRAT

Lanthanzusatz (Mol/l)	*Anteil an Polonium adsorbiert* (%)		
	pH 2.0	*pH 2.8*	*pH 4.0*
0.3	—	49	24
0.03	96	52	27
0.003	78	52	27
0.0003	75	54	35
kein Zusatz	71	73	62

Die Werte für die Aufnahme von Polonium durch Zellulose stellen sich sowohl bei Adsorption als auch bei Desorption ein; d.h. der bei der Adsorption gefundene Wert wird auch erreicht, wenn man zuerst Polonium bei einem bestimmten pH-Wert an Zellulose adsorbiert und die Zellulose dann wieder in einer Lösung desselben pH-Werts suspendiert. Enthalten diese Lösungen Lanthannitrat, so werden bei den Desorptionsversuchen Werte für die Polonium-Verteilung erhalten, die mit denen aus den entsprechenden Adsorptionsversuchen gut übereinstimmen.

Bei den Versuchen mit Glas als Adsorbens zeigten die beiden verwendeten Glassorten weitgehend ähnliches Verhalten, wenn man auf gleiche Oberflächen bezieht. Die Abhängigkeit der Adsorption vom pH-Wert wurde bei zwei Poloniumkonzentrationen untersucht; die Ergebnisse sind in Fig. 2 wiedergegeben. Man sieht, dass bei der niedrigeren Poloniumkonzentration die Adsorption bei einem tieferen pH-Wert ansteigt als bei der höheren Konzentration.

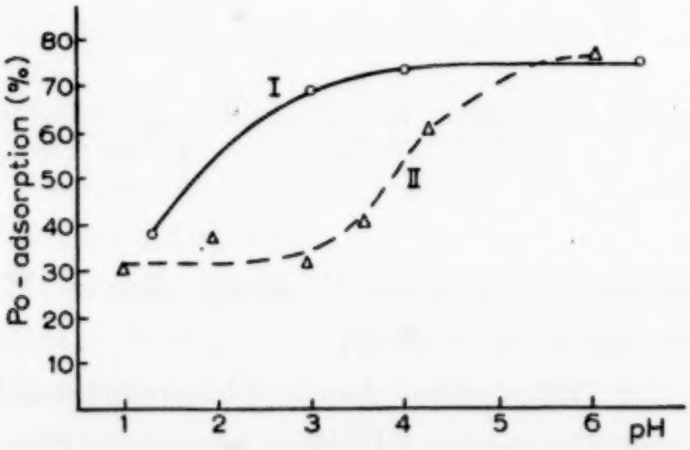

Fig. 2. pH-Abhängigkeit der Adsorption von Polonium an Glas. I: [Po] = $6 \cdot 10^{-11}$. II: [Po] = $3 \cdot 10^{-10}$. Für alle Versuche: [Na$^+$] + [H$^+$] = [Cl$^-$] = 0.1.

Der Einfluss von Lanthannitratzusätzen auf die Adsorption wurde bei einem pH-Wert von 4.0 und einer Poloniumkonzentration von $2.5 \cdot 10^{-10}$ Mol/l untersucht. Im Gegensatz zu den Versuchen mit Zellulose als Adsorbens (bei denen allerdings mit einer geringeren Poloniumkonzentration gearbeitet wurde) wurde dabei eine Steigerung der Adsorption beobachtet.

DISKUSSION

Wenn auch eine umfassende Deutung der hier geschilderten Adsorptionserscheinungen nicht gegeben werden kann, so sollen doch einige Fragen diskutiert werden, die sich in diesem Zusammenhang ergeben. Sicherlich ist die ausgeprägte Neigung des Poloniums zur Bildung von Radiokolloid-Aggregaten für die im untersuchten pH-Bereich auftretenden Adsorptionserscheinungen von Bedeutung. Allerdings existiert noch durchaus kein geschlossenes Bild des Adsorptionsverhaltens von Radiokolloiden; insbesondere scheint noch nicht geklärt zu sein, wann Radiokolloide stark adsorbiert werden und wann anderseits Radiokolloidbildung zu einer Adsorptionsverringerung führt. Auch über den Einfluss von pH und von Salzzusätzen auf die Radiokolloidbildung, z.B. über die Beeinflussung der Grösse der Aggregate und ihrer Ladung, ist wenig bekannt.

Die Adsorption von Radiokolloiden an Filtermedien, insbesondere an Zellulose, bildet die Grundlage von Abtrennungsmethoden für einige trägerfreie Radioelemente (Zusammenfassungen bei[5,6]). Starke Adsorption von Radiokolloiden des Zirkoniums und Niobiums an Kationenaustauschern ist von SCHUBERT UND RICHTER[7] festgestellt worden. Auch die von SCHÖNFELD UND BRODA[8] erhaltenen Ergebnisse über die Adsorption trägerfreien Bleis (Thorium B) an Zellulose in Gegenwart von H_2S, bei denen ein Maximum der Adsorption bei pH 2 festgestellt wurde, können nur als Kolloidadsorption gedeutet werden.

Hingegen haben RYDBERG UND RYDBERG[9] das Auftreten eines Maximums der Adsorption von trägerfreiem Thorium an Glas bei pH 4.5 durch die Annahme gedeutet, dass der Anstieg zum Adsorptionsmaximum (von niedrigeren pH-Werten kommend) auf der zunehmenden Abdissoziation von H^+ von den Kieselsäureendgruppen der Glasoberfläche und einer dadurch bewirkten Zunahme der Ionenaustauschadsorption beruht, während bei noch höheren pH-Werten durch Hydrolyse des Thoriums kolloidale Aggregate gebildet werden und dadurch die Adsorption verringert wird. In ähnlicher Weise haben HAISSINSKY und Mitarbeiter[10] Beobachtungen über die Adsorption von Ce, Pm und Ru an verschiedenen Oberflächen (Platin, Silber, rostfreier Stahl, Polyvinylchlorid) gedeutet, bei denen ebenfalls bei bestimmten pH-Werten ausgeprägte Adsorptionsmaxima aufgetreten waren. Als wirksamer Adsorptionsmechanismus der Ionenadsorption wird hierbei die Gegenionenadsorption an einer primär adsorbierten Schicht von Anionen angesehen, d.h. also eine Aufnahme in die elektrische Doppelschicht der Grenzfläche.

Von den hier mitgeteilte Ergebnissen scheint besonders die Steigerung der Adsorption von Polonium an Zellulose durch grössere Konzentrationen von Lanthannitrat bei pH 2.0 (vgl. Tabelle I) darauf hinzuweisen, dass der Anstieg der Adsorption im Bereich pH $\sim$ 2 auf Kolloidadsorption zurückzuführen ist. Würde in diesem pH-Bereich Ionenaustauschadsorption an den Karboxylgruppen der Zellulose vorherrschen—was schon wegen der schwach sauren Natur dieser Gruppen wenig wahrscheinlich ist[11]—so wäre wohl mit einer beträchtlichen Verringerung der Adsorption durch den Elektrolytzusatz zu rechnen.

Während SCHUBERT UND RICHTER[7] auf Grund ihrer Versuche mit Zirkonium und Niobium zur Auffassung gelangten, dass Elektrolytzusätze die Radiokolloidadsorption verstärken—oder zumindest nicht verringern—, zeigen die hier mitgeteilten Ergebnisse, dass auch Verringerung der Radiokolloidadsorption auftreten kann. Man muss nämlich annehmen, dass die Verringerung der Adsorption an Zellulose durch Lanthannitratzusatz bei pH $\geqslant$ 2.8 auf einer Beeinflussung der Radiokolloidadsorption, aber nicht auf einer Zurückdrängung einer etwa vorliegenden Ionenaustauschadsorption beruht. Wenn Ionenaustauschadsorption eine wesentliche Rolle spielen würde, so wäre zu erwarten, dass 0.1 M $Ba(NO_3)_2$ mindestens ebenso stark verdrängend wirkt wie 0.0003 M $La(NO_3)_3$. Bemerkenswert ist ja auch die Tatsache, dass schon die niedrigsten Konzentrationen an Lanthannitrat eine beträchtliche Adsorptionsverringerung bewirken. Welcher Mechanismus hierfür massgebend ist, kann vorläufig nicht ausgesagt werden. Näher zu überprüfen wäre jedenfalls, ob nicht Lanthan auch schon bei pH-Werten, wo seine Salze selbst nicht merklich hydrolysieren, in Radiokolloidteilchen eingebaut, bzw. an deren Oberfläche adsorbiert werden kann und so eine Änderung des Adsorptionsverhaltens des radiokolloidal vorliegenden Elementes herbeiführt.

Es muss darauf hingewiesen werden, dass die hier vertretene Meinung einer Radiokolloidbildung und Radiokolloidadsorption des Poloniums bei pH-Werten oberhalb etwa 1.5 im Gegensatz zu den Auffassungen anderer Autoren steht, insbesondere zu der von STARIK[4], der für das pH-Bereich 1–4 Polonium in Lösung vorwiegend einem Ionenzustand zuordnet und die in diesem Bereich auftretenden Adsorptionserscheinungen als Ionenaustauschadsorption deutet. Die von uns beobachtete Beeinflussung der Adsorption durch Elektrolytzusätze kann aber mit einer überwiegend auf Ionenaustausch beruhenden Adsorption nicht in Einklang gebracht werden. In diesem Zusammenhang sei auch erwähnt, dass Adsorption von trägerfreiem Wismut (Radium E) oberhalb pH = 1.7 an Anionenaustauschern beobachtet worden ist; diese Erscheinung ist bestimmt als Radiokolloidadsorption anzusehen.

ZUSAMMENFASSUNG

Es werden Ergebnisse über die Adsorption von Polonium an Zellulose und Glas mitgeteilt, bei denen vor allem der Einfluss des pH und von Salzzusätzen ermittelt wurde. Besonders die Wirkung von Salzzusätzen weist darauf hin, dass die Adsorption von Radiokolloidaggregaten als wesentlicher Adsorptionsmechanismus anzusehen ist.

SUMMARY

The results are reported of studies on the adsorption of polonium on cellulose and glass. Especially the influence of pH and of added salts was investigated. The effect of added salts, in particular, indicates that the adsorption of radiocolloidal aggregates must be considered as the essential adsorption mechanism.

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PAPER CHROMATOGRAPHIC IDENTIFICATION OF CYCLIC IMINO ACIDS

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Cyclic imino acids have been found in many plants (for a recent review see FOWDEN[1]) and also in some antibiotic and fungus peptides (*e.g. allo*-hydroxyproline in phalloidin[2] and in etamycin (viridogrisein)[3], γ-methylproline in antibiotic I.C.I. 13959[4]). During the elucidation of the structure of Staphylomycin S, we have isolated a new imino acid, 4-oxo-L-pipecolic acid[5]. In the course of this work, we have studied the chromatographic behavior and the color reactions of many cyclic imino acids. Our results and the pertinent literature data will be presented here.

PAPER CHROMATOGRAPHY

For the separation of a complex mixture of these amino acids, two-dimensional chromatography is necessary. The two usual combinations[6]: butanol–acetic acid/phenol or phenol/collidine–lutidine are satisfactory. We have used the first system, and the position of the amino acids can be seen in Fig. 1. We have confirmed repeatedly the observation[6b] that chromatography with butanol–acetic acid followed by phenol (saturated with water) gives better resolution and smaller spots than does the reverse order. Paper chromatography in one direction will be sufficient for the resolution of a simple mixture of these amino or imino acids as may be deduced from the R_F values given in Table I. The best system in the case of amino acids that are difficult to separate is *tert.*-amyl alcohol–2,6(or 2,4)-lutidine–water[7] (descending, 4 days, Whatman No. 4 paper), and we found that not only the three piperidinecarboxylic acids, but also the two isomers of each of the hydroxypipecolic acids could be separated with this solvent mixture. COHEN *et al.*[8] have observed that in this system 4-hydroxyproline and 5-hydroxypipecolic acid (OH–COOH *trans*) migrate faster than their *allo*-isomer (OH–COOH *cis*). We have confirmed these results and have also found that the isomers of 3- and 4-hydroxypipecolic acids could be separated (Table I). It has also been observed that the normal (*trans*) isomer of hydroxyproline[9,10] and of 5-hydroxypipecolic acid[8,11] is the faster moving component on a Dowex 50 column.

We have obtained partial separations with *sec.*-butanol–88 % formic acid–water (15:3:2)[12] and with *tert.*-butanol–88 % formic acid–water (70:15:15)[7,10] (ascending), but these systems were much inferior.

The solvent mixture: *n*-butanol–pyridine–acetic acid–water, 40:10:10:50

(descending), which is very useful in the chromatography of peptides[5,13], gives a separation of the isomers of 4-hydroxy- but not of 3-hydroxypipecolic acid. With the isoamyl alcohol–pyridine–water system, which permits the resolution of an isoleucine–leucine–norleucine mixture[14,15], it is possible to separate pipecolic acid from nipecotic and also from isonipecotic acid, but not nipecotic from isonipecotic acid. Satisfactory chromatography of these three imino acids and of the leucines is observed with *tert.*-amyl alcohol–lutidine–water (Table I). Separation of the two isomers of 5-hydroxypipecolic acid was obtained with collidine–lutidine (3:1)[16]; we did not examine this system for the other hydroxypipecolic acids.

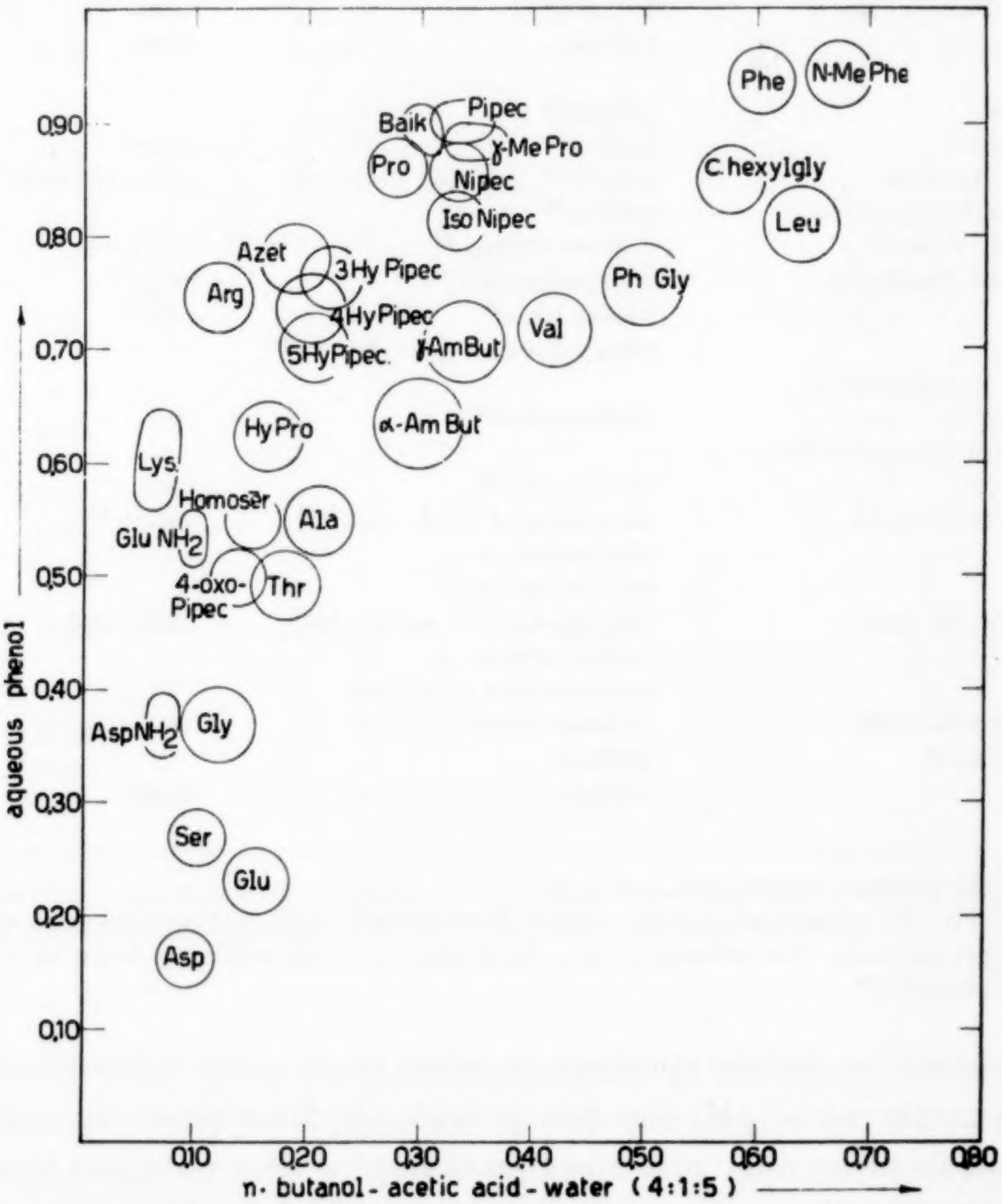

Fig. 1. Pipec = pipecolic acid (2-piperidine-carboxylic acid). Hy = hydroxy (*e.g.* HyPipec = hydroxypipecolic acid). AmBut = aminobutyric acid. Nipec = nipecotic acid (3-piperidine-carboxylic acid). IsoNipec = isonipecotic acid (4-piperidine-carboxylic acid). Baik = baikiain (4,5-dehydropipecolic acid). Azet = 2-azetidine-carboxylic acid. PhGly = phenylglycine (α-amino-phenylacetic acid). N-MePhe = N-methyl-phenylalanine. The generally accepted abbreviations are used for the common amino acids.

The two isomers of the hydroxypipecolic acids are not separated in this system. But their identification is easier in this way than with the solvents of Piez *et al.*[10], which give six spots which are only partially separated. The differences of color obtained with ninhydrin after phenol are also most helpful (see text).

E. Gross (cited in ref. [17]), has separated *allo*-hydroxyproline from hydroxyproline by high voltage electrophoresis (50 V/cm, 75 cm strip) in a buffer of pH 1.9[18].

TABLE

Compound	Ninhydrin color	Fluorescence of ninhydrin color with U.V. light	Isatin[11]
2-Azetidine-carboxylic acid	brown[26]	none	decolorization[1]
Proline	yellow	none	blue[21]
4-Methylproline	yellow[27]		blue[27]
4-Hydroxyproline	yellow	none	blue
Hydroxymethylproline	yellow[25], pinkish yellow[28]	salmon-pink[28]	blue[25, 28]
4-Hydroxy-4-methylproline	yellow[29]		
5-Hydroxymethyl-proline	yellow-orange[11]	brick-red[11]	
Δ^4-Pipecolic acid (baikiain)	yellow-brown[30, 31]	none[31]	yellow-brown[31]
Pipecolic acid	violet-blue[27] violet-blue → gray-brown[32],	red[31]	blue[27, 32]
Nipecotic acid (3-piperidine-carboxylic acid)	violet-blue[32]	red	neg.
Isonipecotic acid (4-piperidine-carboxylic acid)	violet-blue[32]	red	neg.
5-Hydroxypipecolic acid	blue tinged with violet[33], red-violet → yellow-brown[31]	red[11, 31]	blue[31, 33]
4-Hydroxypipecolic acid	orange-brown → purple[34], yellow-green → gray-purple → yellow	faint red	blue
3-Hydroxypipecolic acid	yellow-blue[34], yellow	none	neg.
4-Oxopipecolic acid	yellow		neg.
Alanine	violet	none	neg.

* In phenol–water (pH 5.0–5.5).

** The R_F values of 4- and 5-hydroxypipecolic acid in these solvent systems are those of the normal isomers. The structure of 4-hydroxypipecolic acid has been in doubt[34], but has now been confirmed[35, 36].

We could separate the hydroxyprolines by usual paper electrophoresis (4 V/cm, 17 h, 40 cm strip) at the same pH, but not 3- and 4-hydroxypipecolic acid. Resolution might be possible at another pH. Since good results were obtained in all cases with *tert.*-amyl alcohol–lutidine, this system was not studied further.

COLOR REACTIONS

The amino acids may be identified by their location on the chromatogram, supplemented by co-chromatography of the authentic amino acid and those adjacent to it. But support of this identification by color reactions is very useful.

The description of the color and color evolution obtained after reaction of the piperidinecarboxylic acids with ninhydrin differs somewhat between different authors. This is due partly to differences in the definition of the shade observed (purple–violet–blue), partly to the composition of the ninhydrin reagent, the temperature and duration of heating, and the presence of residues of the solvent used during the chromatography (lutidine and collidine are often responsible). We have often used a ninhydrin reagent (0.1 % in ethanol) containing 2,6-lutidine (2 drops per 100 ml), and have found that the different shades observed during the evolution of the color (generally

I

Kolor-Roberts[22]	*Plattner-Nager*[23]	R_F			R_{Ala}*** *tert.-AmOH–2,4-lutidine–water (178:178:114)*
		n-BuOH–AcOH–H₂O (4:1:5)	*Phenol–NH₃ soln.*	*Collidine–lutidine (1:3)*	
neg.		0.20[26]	0.80–0.84[26]		0.77
yellow orange → red[22]	neg.	0.30[27]	0.92[27]		1.25
		0.44[27]	0.88[27]		
red-purple[22]	neg.	0.19[27] 0.25[28]	0.65[27]		1.04;0.83[8]
neg.			0.878[32]*	0.374[32]	1.65
rose	rose	0.42[27]	0.92[27] 0.895[32]*	0.384[32]	1.88
neg.	neg.		0.882[32]*	0.270[32]	0.68
neg.	neg.		0.878[32]*	0.254[32]	0.30
red-purple → violet	rose	0.254[33]	0.783[33] 0.744[31]*	0.374[31]	1.31;1.00[8]
red-violet → gray-purple	rose	0.232[33]**	0.826[33]**		1.27;0.87
yellow	orange				1.67;0.95
neg.	neg.				0.75
		0.247[33]	0.609[33]		1.00

*** R_{Ala} = migration relative to alanine. Of the two values of R_{Ala} given for the hydroxy imino acids, the first refers to the normal (OH–COOH *trans*), the second one to the *allo*-isomer (OH–COOH *cis*).

violet to yellow) were useful for the identification of the piperidinecarboxylic acids. The ninhydrin color of many of these imino acids produced a red fluorescence in ultraviolet light (365 mμ) (Table I). When sprayed with copper nitrate solution[19], the ninhydrin spot of these cyclic imino acids did not turn to red, as is observed with the amino acids. This is not unexpected as the structure of the dye is not favorable for the formation of a complex with copper[20].

It should be noted that no fluorescence of the ninhydrin spots was observed when phenol was the last solvent used on the chromatogram. In this case the ninhydrin color of some imino acids was also different which was very useful for their identification: 3-hydroxy-(yellow), 4-hydroxy-(greenish blue), 5-hydroxypipecolic acid (violet), baikiain (yellow green). The isatin reagent[21] (0.2 % in butanol or acetone containing 4 % acetic acid) was also used. The blue color appears more slowly with many cyclic imino acids than with proline (it is sometimes necessary to keep the paper for one day in the dark in order to obtain full intensity). *allo*-Hydroxyproline gives the same color as hydroxyproline. This reaction, performed in solution, has been described as negative for *allo*-hydroxyproline[2].

The color obtained with ROBERTS–KOLOR reagent (ninhydrin–isatin–triethylamine)[22] is very useful for identification. The PLATTNER–NAGER reagent (*p*-nitrobenzoyl chloride–pyridine) is noted here because it gives a specific color (rose) with N-alkyl-amino acids[23] *cf.* [5]. It is much less sensitive for cyclic imino acids (some 100 µg per spot are necessary).

The test of JEPSON AND SMITH[24] apparently is specific for hydroxy- and *allo*-hydroxyproline. It is negative for hydroxymethylproline[25], for the piperidinecarboxylic acids, and the hydroxypipecolic acids.

ACKNOWLEDGEMENTS

We wish to thank Dr. H. C. BEYERMAN (Delft), Dr. L. FOWDEN (London) and Professor R. A. RAPHAEL (Glasgow) for samples of 5-hydroxypipecolic acid, azetidine-carboxylic acid and baikiain.

SUMMARY

The paper chromatography of cyclic imino acids in several systems has been studied. The color reactions, which may also be helfpul in their identification, have been examined.

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CONTINUOUS ZONE ELECTROPHORESIS OF PITUITARY EXTRACTS

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INTRODUCTION

In a study of the pituitary factors influencing wool growth in the sheep, a fractionation procedure was sought by which pituitary hormones could be prepared in their native states from the same batch of starting material and as free as possible from cross contamination.

Fractionation schemes using chemical methods[1-3], have not resulted in clean separations of pituitary hormones and continuous zone electrophoresis was applied to this problem.

Several investigators[4-13] have described methods for the continuous zone electrophoresis of proteins.

MATERIALS AND METHODS

(*a*) *Apparatus*

The continuous electrophoresis apparatus used (supplied by Shandon Scientific Co., London) is similar to that described by HOLDSWORTH[13]. Preliminary investigations showed that the apparatus, in its original form was unsuitable for preparing the amounts of material required for testing in sheep, and it was modified.

A larger power supply was provided and continuous electrode flushing was achieved by using a metering pump. Test tubes were replaced by 250 ml collecting bottles. The protein was injected by a mechanically controlled syringe either directly on to the paper or via a small container and paper wick. Two different supporting media were used, one a single thick cellulose filter pad and the other several sheets of filter paper sewn together. All separations were carried out at 0°.

(*b*) *Buffer*

The buffer contained 0.025 N lithium hydroxide and 0.1 M boric acid (pH 8.6 and ionic strength 0.025).

(*c*) *Pituitary extract*

Deep-frozen sheep pituitary glands were ground and extracted with a pH 8.6, 0.05 ionic strength lithium borate buffer containing 0.45 M NaCl. The extract was dialysed and freeze dried.

(*d*) *Protein concentration*

This was determined by measuring the absorption of the solution at 280 mμ[14], and also colorimetrically[15]. In some runs material was weighed after dialysing and freeze drying the solutions collected.

(*e*) *Electrophoretic analysis of fractions*

The fractions obtained were analysed electrophoretically on paper strips in pH 9.4, 0.05 ionic strength lithium borate buffer.

The apparatus used was a modification of that described by VALMET AND SVENSSON[16]. A gradient of pH, viscosity and ionic strength was used to improve the resolution which is generally poor with pituitary proteins using conventional methods. Electrophoresis was carried out for 9 h at a voltage of 15.5 V/cm and a strip temperature of 6–9°. The dried strips were stained with Naphthalene Black 12B.

The stained strips were photographed and a × 2 enlargement made on positive film using a standardized procedure. The enlargements were scanned in a photoelectric densitometer (Evans Electroselenium Ltd., Harlow, Essex).

Preparations of ox growth hormone (lot R50109) and sheep prolactin (lot R10109) prepared by Armour Laboratories, Chicago, U.S.A. and kindly supplied by the Endocrinology Study Section, National Institutes of Health, U.S.A. were used as electrophoretic standards.

(*f*) *Biological assay methods*

Growth hormone was determined by the increase in body weight of hypophysectomised rats[17,18].

Gonadotrophic activity was determined by the hyperaemic response of the ovaries of immature female rats. This was based on the method of SALMON, GEIST, SALMON AND FRANK[19].

Adrenocorticotrophic hormone was assayed by the method of SAYERS, SAYERS AND WOODBURY[20].

Thyrotrophic hormone was determined by the increase in weight of the thyroids of day-old chicks[21,22].

RESULTS

Stability of separation

Fig. 1 shows the distribution of protein in five separate collections from an experiment running continuously for 133 h. 2 g of protein were collected over this period. The pattern remained reasonably stable but as shown below, the various fractions do not represent discrete separation of the component proteins.

Examination of fractions by paper strip electrophoresis

Figs. 2 and 3 show photographs of the stained strips together with the distribution of components obtained by scanning × 2 enlargements of the strips on positive film. The proportion of the total protein in each fraction, based on the weight of freeze-

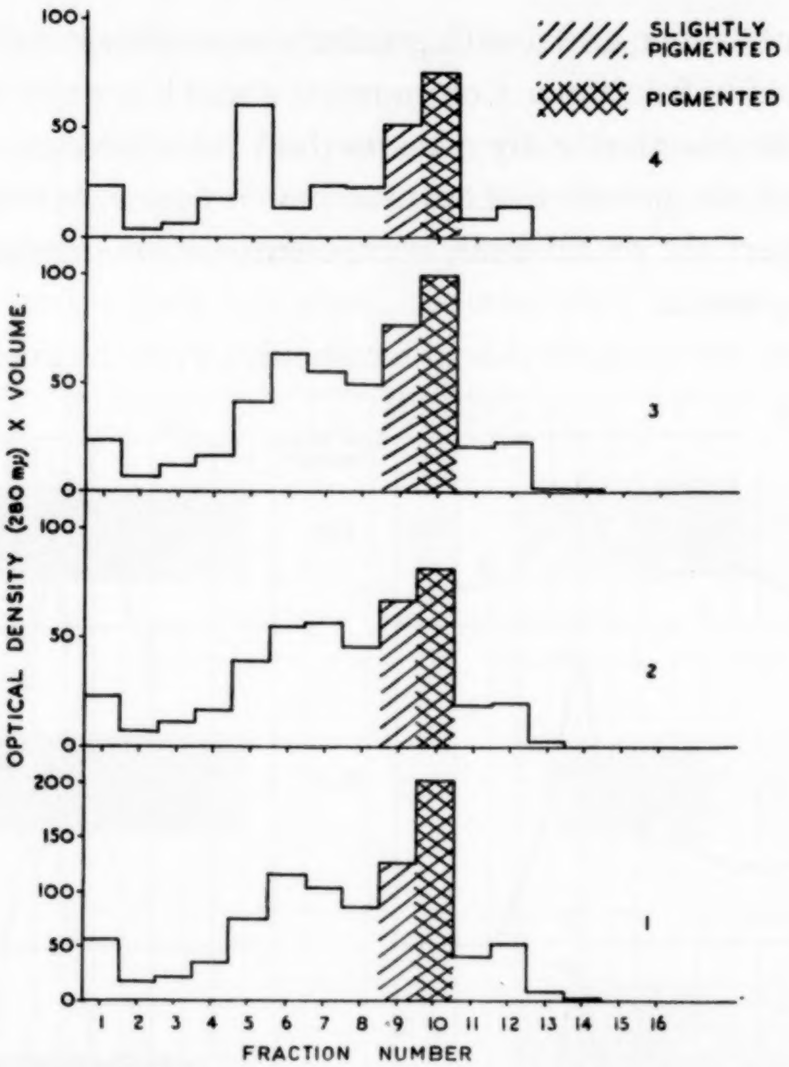

Fig. 1. The distribution of protein in the collecting bottles for 4 separate collections from an experiment running continuously for 133 h (lithium borate buffer, pH 8.5, $\mu = 0.025$, 520 V, 79 mA, 2.5°; 8 sheets Whatman No. 1).

dried material is also shown. Also included are strips of ox growth hormone and sheep prolactin standard preparations.

Inspection of the stained strips showed the presence of at least 12 separate electrophoretic components*. For convenience of description these components have been allotted the letters a to l, beginning with the component nearest the anode.

Components i and j were identified as sheep haemoglobins A and B, and component e as albumin. Most of this albumin in the extract probably arose from contamination with serum proteins. Comparison of components i and j with standard sheep haemoglobin showed that the intensity of the pigment in these components was not sufficient to account for the amount of protein present as estimated by the intensity of the staining with Naphthalene Black. Components i and j must therefore each consist of two or more electrophoretically similar components.

Inspection of Figs. 2 and 3 shows that each fraction contained at least three components and each component was present in more than one fraction.

Such a wide spread of each component in the continuous apparatus was not found with plasma proteins and was attributed to protein interactions between the components.

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* Additional components have been found after starch gel zone electrophoresis. These results will be described in another paper.

Four components a, b, c and d with greater electrophoretic mobility then albumin were clearly identified in fraction 2. Components a and b are not shown in the diagram. Gaussian analysis of fraction 2 by the method of HOXTER, WAJCHENBERG AND MUNGIOLI[24] indicated the presence of two further components between d and albumin. There are then at least six prealbumin components. None of these could be detected in runs with sheep plasma.

Component k, on the cathode side of h coincides with the major peak of the growth

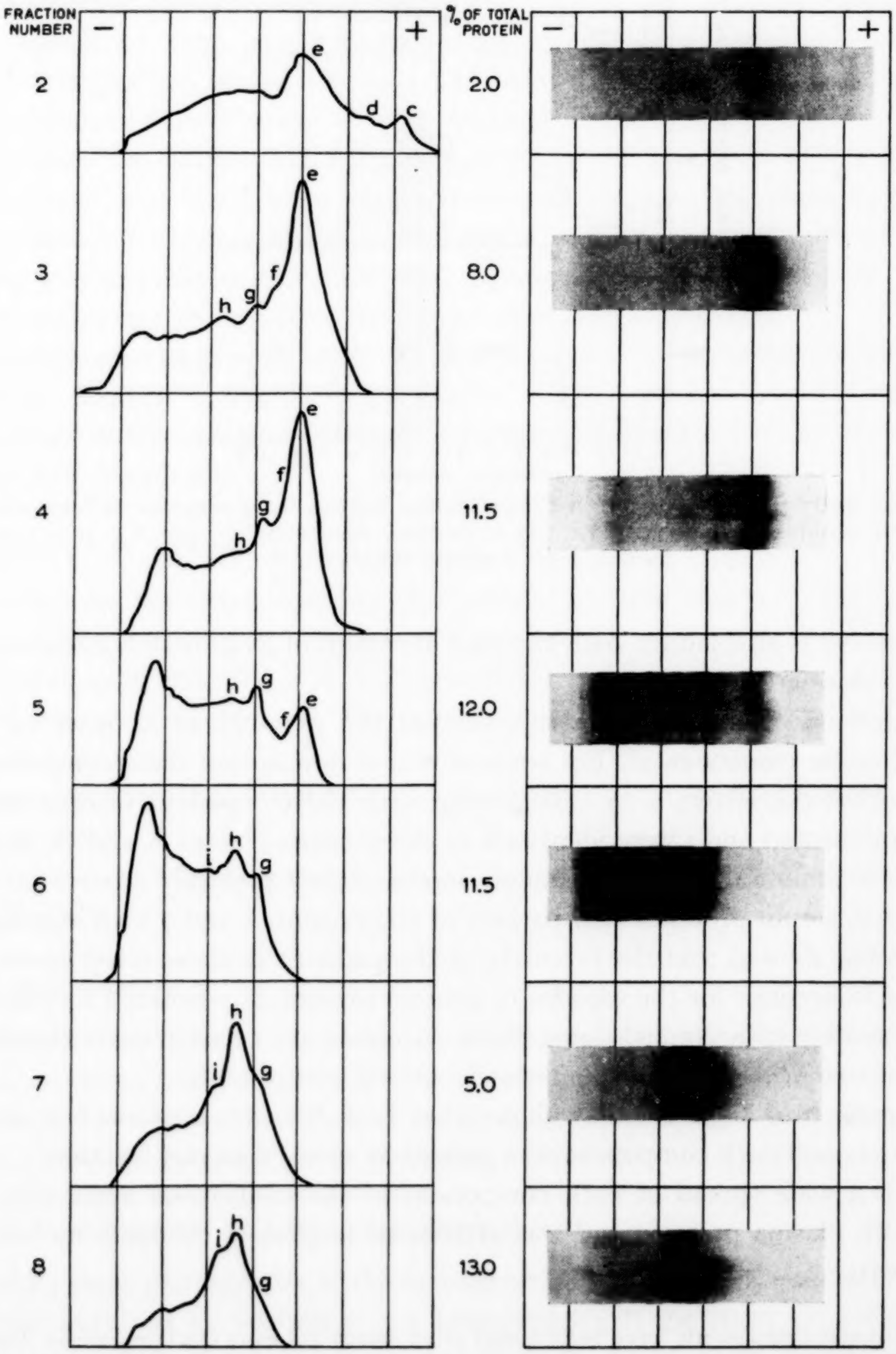

Fig. 2. Paper strip electrophoresis of fractions 2 to 8 obtained by continuous electrophoresis.

hormone standard. The results from biological assay support the identification of k as growth hormone.

The two major components of the prolactin standard coincide in position with j, the slower pigmented component. It is possible then that prolactin accounts for some of the protein material in j which is not accounted for by haemoglobin.

Fractions 3, 4, 5 and 6 show adsorbed material close to the origin. This material

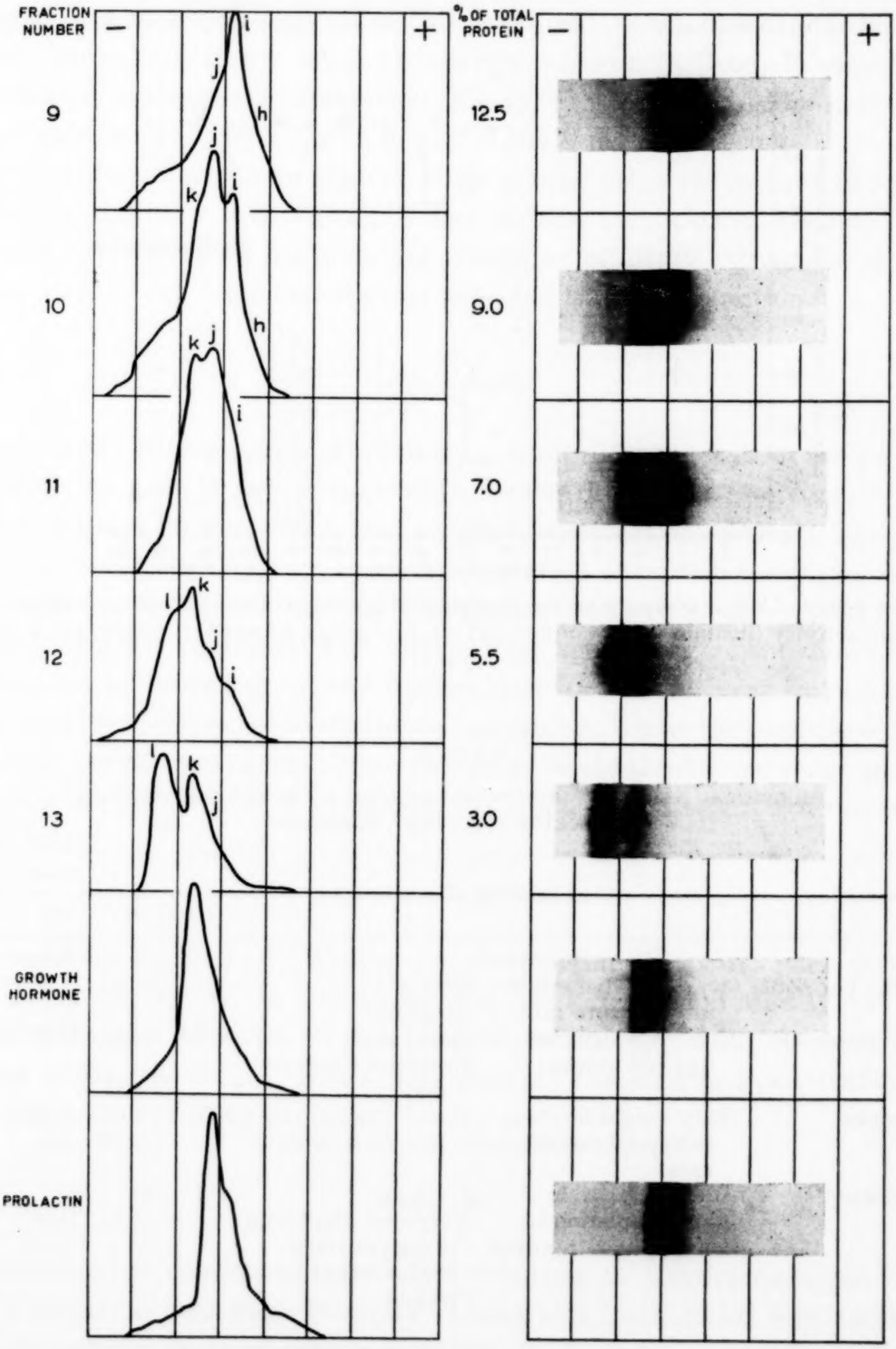

Fig. 3. Paper strip electrophoresis of growth hormone and prolactin standards and fractions 9 to 13 obtained by continuous electrophoresis.

presumably represents one or more components with a true mobility close to that of g. Adsorption to the paper does not affect the mobility of components in the continuous apparatus and this possibly accounts for the presence of this material in the faster fractions.

Biological assay of hormone activity after separation

A preliminary biological assay of hormone activity in the various fractions has been carried out. The material was obtained in the earlier experiments by separating crude

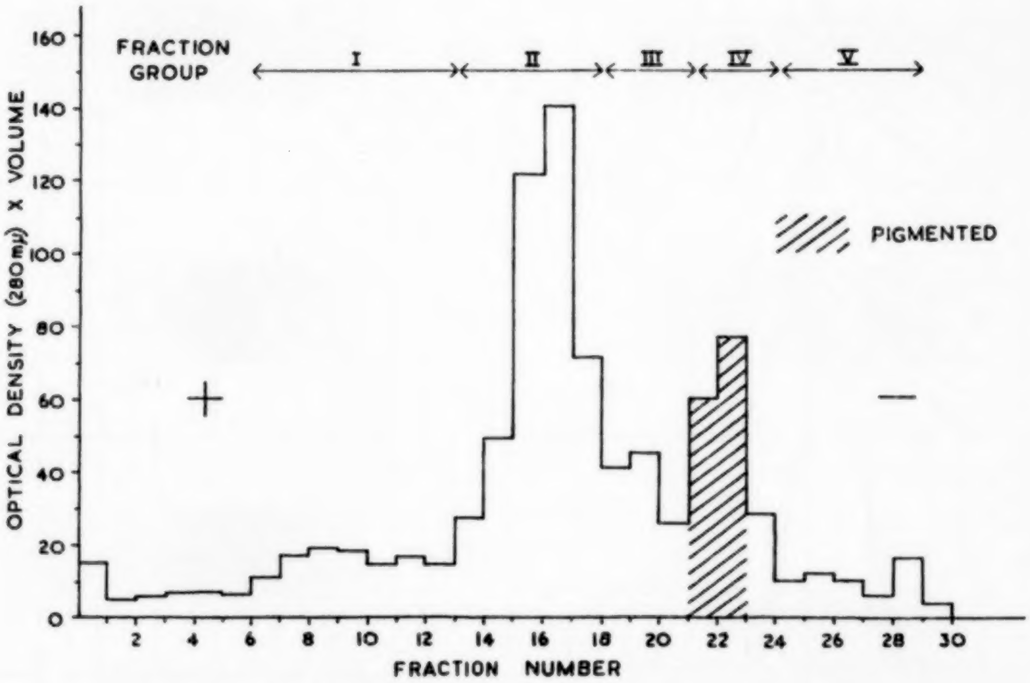

Fig. 4. The distribution of protein and the grouping of fractions in the collection used for the assay of hormone activity (lithium borate buffer, pH 7.1, $\mu = 0.025$, Ekwip D.S. paper, 400 V, 65–70 mA, 5.0°).

TABLE I

BIOLOGICAL ASSAY OF GROUPS OF FRACTIONS FOR THE PRESENCE OF ANTERIOR PITUITARY HORMONES

Hormone	*Test response*		*Group*				
			I	*II*	*III*	*IV*	*V*
Gonadotrophic hormones	Ovarian hyperaemia in immature rats	Dose mg	1.5	2.9	N.T.	N.T.	1.5
		Response	—	+			—
Thyrotrophic hormones	Thyroid weight in day-old chicks	Dose* mg	N.T.	5.0	N.T.	5.0	N.T.
		Response (increase mg)		4.4§		2.3	
Growth hormone	Body weight increase in hypophysectomized rats	Dose** mg	N.T.	0.6	0.65	0.65	N.T.
		Response (g/day)		0.5§§	0.3	1.4§	
Adrenocorticotrophic hormone	Adrenal ascorbic acid depletion in hypophysectomized rats	Dose μg	N.T.	30	65	65	N.T.
		Response (depletion in mg per 100 g fresh adrenal tissue)		— 5.8	19.0	0.4	

* Total dose over 3 days.
** Daily dose for 8 days.
§ $P < 0.001$.
§§ $P < 0.01$.

pituitary extract on a filter pad. The spread of fractions, (Fig. 4) was not as great as in the later experiments, *cf.* Fig. 1. Components e to l occurred between fractions 14 to 24 inclusive. To reduce the number of assays necessary, the collected fractions were combined into 5 groups labelled from the anode I to V (Fig. 4).

The hormone activities of the groups are given in Table I. All groups have not been tested for all activities.

Both gonadotrophic and thyrotrophic activity is associated with Group II. The slight growth-stimulating activity of this group is possibly due to the presence of these two hormones. Growth hormone activity is shown by Group IV supporting the electrophoretic evidence that component k is growth hormone. Adrenocorticotrophic activity could not be demonstrated in any of the three major groups.

Investigation of prolactin content of the groups using the method of LYONS[25] as modified by BRADLEY AND CLARKE[26] has yielded inconclusive results. However, continuous electrophoretic fractionation of another pituitary extract has shown that prolactin activity can be obtained at its expected isoelectric position.

DISCUSSION

The continuous electrophoresis of pituitary extract did not result in the preparation of any protein in pure form in a single stage. In this respect the resolving power of the method is perhaps no worse than that of other fractionation methods. The difficulty common to all methods appears to be the formation of protein complexes which are incompletely broken down either by electrophoretic force or differential solubility. Analytical strip electrophoresis with high voltage against a gradient shows that such complexes can be broken down and further experiments are being carried out in an attempt to achieve this with the continuous apparatus. Under the conditions described considerable purification of some hormones could be obtained in one stage and further purification has been achieved by passing particular fractions through the apparatus a second time.

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SUMMARY

The application of continuous zone electrophoresis to the fractionation of sheep pituitary extract is described. Pituitary extract was fractionated into 12 fractions. Each contained 3, 4 or 5 components and each component was common to more than one fraction. It is suggested that the formation of protein complexes prevents resolu-

tion into mutually exclusive fractions. Preliminary evidence is given on the distribution of hormone activity.

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A COMBINED DETECTING REAGENT FOR THE IDENTIFICATION OF ORGANIC ACIDS ON PAPER CHROMATOGRAMS

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The separation of mixtures of organic acids by means of descending and ascending paper chromatography is very difficult, because the R_F values of some of the acids are very similar in most of the solvent systems. In order to eliminate this deficiency to a certain extent many authors combine at least two systems of developing solvents, or they use two-dimensional chromatography.

The specific detecting reagents that are used for the identification of organic acids are, in general, based upon the different pH values of the acid spots being detected and of the background. For this purpose the following acid–base indicators are most successful: bromocresol green, bromophenol blue, bromothymol blue, bromocresol purple, etc. The advantage of these reagents is their sensitivity and the constancy of the spots. Alkaline or acidic $KMnO_4$ solutions, which have a rather wide detection range, differentiate organic acids from other organic materials by different spot colours[1–5].

It was found disadvantageous that most of the reagents that are suitable for detecting organic acids produce the same colour with all the acid spots, which makes the identification of acids with similar R_F values difficult.

The combination of acid–base indicators with alkaline $KMnO_4$ solution has enabled us to propose a new detecting reagent, which yields different colours with different organic acids and so makes their identification easy.

EXPERIMENTAL

Preparation of the reagent

(1) 0.075 % bromocresol green + 0.025 % bromophenol blue in absolute ethyl alcohol.

(2) 0.5 % $KMnO_4$ + 1 % $Na_2CO_3 \cdot 10H_2O$ in distilled water.

Both solutions are stable. Shortly before detection of the chromatograms the required quantities of both solutions are mixed in the ratio 1:1. Immediately after mixing the solutions it is necessary to spray the chromatograms because this detecting reagent is only stable for 5–10 min. The colours of the spots of the separate acids obtained with this reagent are given in Table I.

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TABLE I

R_F VALUES OF ORGANIC ACIDS AND COLOURS OBTAINED AFTER SPRAYING WITH REAGENT

Organic acid	R_F	*Colour*	*
Gluconic acid	0.06	light grey-blue	C
2-Ketogluconic acid	0.08–0.1	yellow	B
5-Ketogluconic acid	0.15–0.17	orange-red	B
Tartaric acid	0.22–0.26	purple-red + violet edge	B
Oxalosuccinic acid	0.25–0.33	red-orange + purple edge	B
Citric acid	0.36–0.4	violet + yellow-rose centre	B
Isocitric acid	0.39–0.42	white-orange + violet edge	B
Malic acid	0.45–0.52	yellow-orange + violet edge	B
Malonic acid	0.59–0.65	orange + purple edge	A
Pyruvic acid	0.65–0.75	dark violet	B
Lactic acid	0.64–0.7	red-orange + red edge	B
Succinic acid	0.71–0.73	orange-red + red edge	A
Aconitic acid	0.73–0.77	yellow + green edge	B
Itaconic acid	0.77–0.82	green + yellow centre	A
Fumaric acid	0.82–0.85	yellow + green edge	A
Adipic acid	0.82–0.84	red	A
Oxalic acid	a prolonged spot	purple	B
α-Ketoglutaric acid	a prolonged spot	violet	B

* The background of the chromatogram is grey-blue. A: the spot appears during the spraying of the chromatogram. B: the spot appears within 30 sec after spraying. C: the spot appears 2–3 min after spraying.

Method

The organic acids were separated by one-dimensional paper chromatography with the descending method, using Whatman No. 1 filter paper sheets of 33 × 18 cm; the solvent system was *n*-butanol–formic acid–water (10:1:5) and chromatography was carried out in glass-jars in the usual manner. 150 μg amounts of aqueous solutions were placed 9 cm from the bottom edge of the paper sheet. The developing time at room temperature was 18 hours. The chromatograms were then dried at room temperature in a stream of air for 4–6 hours.

DISCUSSION

Optimal coloration of the spots occurs within 2–5 minutes after spraying of the chromatogram; it is most advantageous to carry out the evaluation then, since later on the spots lose their characteristic colour, although they remain permanently visible. When detecting acids at lower concentrations, the surface of the spot is smaller so that the outer colouring of the spots obscures the colouring of the centre. On the other hand, when detecting individual acids at higher concentrations, the colour of the spot centre predominates over that at the edge.

This reagent has proved very useful for the identification of organic acids produced by *Aspergillus niger* mould in fermentation media of various compositions.

By means of this combined detecting reagent it was possible, to trace the organic acids in the sample without their preliminary isolation because the changes in the R_F values caused by the presence of various extraneous materials or by the unequal concentration of the acids studied does not interfere with their identification.

SUMMARY

A combined detecting reagent consisting of a mixture of acid–base indicators with alkaline $KMnO_4$ solution is described. This reagent facilitates the identification of organic acids on paper chromatograms not only of those with different R_F values, but also of those with similar R_F values because it gives colours that are characteristic for each individual acid.

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ANALYSIS OF PHENYLALKANES BY GAS CHROMATOGRAPHY

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INTRODUCTION

Gas chromatography has been successfully used for the analysis of lower molecular weight hydrocarbons, but only in a few cases has it been employed for hydrocarbons with more than ten carbon atoms. Higher molecular weight materials, in general, have not been analyzed because of the large number of isomers. The lack of known standard compounds has made identification difficult. Gas chromatography has been successfully applied to the analysis of higher molecular weight materials for cases in which the number of isomers is limited. Examples include the methyl esters of fatty acids[1], substituted biphenyls[2], paraffin types in wax[3], and others.

This procedure for the analysis of phenylalkanes is applicable to materials with carbon numbers of C_{12} to C_{20}. Only compounds with a single phenyl group attached to a straight chain alkyl group are considered. There are 104 possible phenylalkane isomers in this molecular weight range. The number of compounds may seem large at first glance but is actually not unmanageable since the largest number of isomers for any one molecular weight is ten. It is also small when compared with the number of isomers in the paraffin series. There are, for example, 36,797,588 possible C_{25} paraffin isomers[4] alone.

Although the number of isomers is small, the physical and chemical properties of these compounds are quite similar. This has made analysis of mixtures difficult. Infrared and mass spectrometers have been used for the analysis of phenylalkanes, but quantitative results are more difficult to achieve than with gas chromatography. Distillation at reduced pressures has been used to separate single carbon number fractions.

The boiling points of the isomers of phenylalkanes of a single carbon number decrease as the phenyl group is moved toward the center of the alkyl chain. Table I lists the boiling points for the phenyldodecanes[5] and the phenyleicosanes[6]. 4-Phenyldodecane is the only compound of these two series which increases in boiling point as the phenyl group is moved toward the center of the chain.

EXPERIMENTAL

A column solvent which has a low vapor pressure at temperatures in excess of 300° is necessary for high temperature gas chromatography operation. The column solvent

TABLE I

BOILING POINTS OF PHENYLDODECANES[5] AND PHENYLEICOSANES[6]

	Boiling point, °C a reduced pressures	*Boiling point, °C* converted to 760 mm Hg*
1-Phenyldodecane	166.1–166.7 at 5 mm	328
2-Phenyldodecane	154.0–154.4 at 5 mm	312
3-Phenyldodecane	172.2–176.1 at 15 mm	311
4-Phenyldodecane	164 –166 at 10 mm	314
5-Phenyldodecane	155 –161 at 10 mm	304
6-Phenyldodecane	144.4–148.3 at 5 mm	304

	*Boiling point, °C** at 760 mm Hg*
1-Phenyleicosane	419
2-Phenyleicosane	(416)
3-Phenyleicosane	(413)
4-Phenyleicosane	(410)
5-Phenyleicosane	(407)
7-Phenyleicosane	(406)
9-Phenyleicosane	(406)

* Converted to 760 mm Hg from midpoints of boiling ranges using nomographs in ref.[6].
** Boiling points in parentheses are converted to 760 mm from measurements at lower pressures.

used for the analysis of alkylbenzenes was prepared from a 92-penetration asphalt of a Middle Eastern crude. The lower boiling asphaltic materials were removed by bubbling nitrogen through the asphalt, at a temperature of 320° for 24 h. The residue obtained from this treatment was deposited on 42–60 mesh Johns-Manvill C-22 firebrick in a ratio of 40:100. A 12-ft., ¼-in. O.D., stainless steel column containing 42 g of this packing was used for all experimental work.

An asphalt column operating at 240° was used for the analyses of phenylhexanes to phenylpentadecanes and at 270° for phenyldodecanes to phenyleicosanes. Several runs were also made at 320°. Resolution at this temperature is poor compared to the two lower temperatures. Helium flow rate in all cases, as measured by a soap bubble flowmeter at the column exit, was 35 ml/min.

Each of the phenylalkane isomers used for gas chromatography standardization was prepared by standard procedures[5]. Identification was established by infrared and mass spectrometer analyses.

DISCUSSION

Retention times, as measured from the air peak, were determined for each of the phenyldodecanes. The order of emergence of the phenyldodecane isomers in order of increasing retention times is 5- and 6-phenyldodecane which are not separated, 4-phenyldodecane, 3-phenyldodecane, 2-phenyldodecane, and 1-phenyldodecane. A phenyldodecane mixture was also analyzed and was found to contain all compounds except the 1-phenyldodecane. Qualitatively, this agreed with mass spectrometer

TABLE II

RETENTION TIMES OF n-ALKYLBENZENES AT THREE TEMPERATURES

Attachment position of phenyl group to n-alkyl group	*Column temperatures, 240°*						*Column temperatures, 270°*						*Column temperatures, 320°*					
	1	*2*	*3*	*4*	*5*	*6 or more internal substitution*	*1*	*2*	*3*	*4*	*5*	*6 or more internal substitution*	*1*	*2*	*3*	*4*	*5*	*6 or more internal substitution*
Number of carbons in n-alkyl group	*Times in min from air*						*Times in min from air*						*Times in min from air*					
-C_6	8.2	5.9	5.1	+	+	+	—	—	—	+	+	+	—	—	—	+	+	+
-C_7	11.6	8.3	7.2	6.3	+	+	—	—	—	—	+	+	—	—	—	—	+	+
-C_8	16.5	11.8	10.2	9.0	+	+	—	—	—	—	+	+	—	—	—	—	+	+
-C_9	23.4	16.7	14.1	12.5	11.8	+	—	8.6	7.5	6.8	6.4	+	—	—	—	—	—	+
-C_{10}	33.1	23.8	20.0	17.8	16.7	+	—	11.7	10.2	9.2	8.6	+	—	—	—	—	—	+
-C_{11}	46.8	33.1	28.2	25.2	23.7	23.7	—	16.6	13.9	11.7	11.7	11.7	—	—	—	—	—	—
-C_{12}	66.2	47.4	39.9	35.6	33.1	33.1	—	22.3	18.6	16.6	15.4	15.4	—	—	—	—	—	—
-C_{13}	95	62.8	56.3	50.0	47.4	45.7	—	29.1	25.1	22.3	21.7	21.7	—	—	—	—	—	—
-C_{14}	133	94.8	79.1	70.4	66.0	63.4	—	40.0	33.9	30.3	28	27.3	—	9.7	8.8	6.9	6.9	6.9
-C_{15}	188	135	112	100	96	87	—	55.2	46.4	41.0	38.4	36.8	—	12.4	10.9	9.7	8.8	8.8
-C_{16}	—	192	156	142	135	120	—	73.5	62.1	55.3	51.3	49.3	—	14.4	13.9	12.4	10.9	10.9
-C_{17}	—	—	220	202	192	168	—	101	84.6	74.6	69.8	66.8	—	18.7	15.3	14.4	13.9	13.9
-C_{18}	—	—	—	—	—	—	—	134	113	94	88.7	88.7	—	23.9	20.8	18.7	15.3	15.3
-C_{19}	—	—	—	—	—	—	—	183	157	136	127	120	—	30.3	26.3	23.9	20.8	20.8
-C_{20}	—	—	—	—	—	—	—	244	208	183	170	157	—	37.0	32.0	30.3	26.3	26.3
RETENTION TIMES OF n-PARAFFIN STANDARDS																		
n-C_{10}	1.9						—						—					
n-C_{11}	2.8						—						—					
n-C_{12}	4.0						—						—					
n-C_{13}	5.8						—						—					
n-C_{14}	8.5						—						—					
n-C_{15}	12.3						6.4						1.9					
n-C_{16}	17.7						8.8						2.5					
n-C_{17}	25.8						12.0						3.2					
n-C_{18}	37.0						16.6						4.1					
n-C_{19}	52.0						22.4						5.4					
n-C_{20}	77						31.0						6.9					
n-C_{21}	—						42.6						9.1					
n-C_{22}	—						58.0						11.8					
n-C_{23}	—						80						15.3					
n-C_{24}	—						110						20.0					

+ An impossible compound.

and infrared analyses. The retention times for the other mixtures of single carbon number phenylalkanes from phenylhexane to phenyloctadecane were also determined. Names were assigned to the peaks in the chromatogram assuming the same retention time order holds for each single carbon number as for the phenyldodecanes. Plots of logarithm of retention times against the number of carbon atoms for each homologous series of phenylalkanes were made. A straight line was obtained for each series. JAMES AND MARTIN[1] showed in their original publication that plots of logarithm of retention time against carbon number for homologous series of iso- and normal fatty acids yielded straight lines. RAY[7] and others have since shown that this relationship holds for other homologous series of organic compounds. Phenylalkanes also appear to show this relationship.

The retention times of phenylalkanes from phenylhexane to phenyleicosane are listed in Table II. In addition, retention times of several normal paraffins are also given as reference standards[8]. Retention times for many of these compounds are listed at 240°, 270° and 320°. All isomers contained in a single molecular weight mixture from phenylhexane through phenyldecane can be separated. Isomers in phenylundecane through phenyltetradecane single molecular weight mixtures are all separated except the 5- and more internally substituted isomers. The 6- and more internally substituted phenylalkanes are not separated in the phenylpentadecanes through the phenyloctadecanes.

For mixtures of phenylakanes of two or more consecutive carbon numbers, certain isomers of each molecular weight are not resolved. In runs made at 240°, the 2-phenylalkanes are not resolved or only partially resolved from the 5-phenylalkane of the next higher carbon number; while runs made at 270° place the 2-phenylalkanes with the 4-phenylalkane containing one more carbon.

Quantitative measurements were made on three mixtures of weighed amounts of pure phenyldodecanes. Results of these analyses are given in Table III. By assuming the areas under each peak are proportional to the weight per cent of the compound present, it is seen that high results (5 % of the amount present) are obtained for 5- and 6-phenyldodecane, and low results (8 % of the amount present) for 4-phenyldodecane. This is probably due to different response factors for each isomer. The accuracy of the analysis can be improved by determining these response factors.

TABLE III

QUANTITATIVE RESULTS OF MIXTURES OF PHENYLDODECANES

Compound	*Mixture 1*		*Mixture 2*		*Mixture 3*	
	Known wt. %	*Analysis area %*	*Known wt. %*	*Analysis area %*	*Known wt. %*	*Analysis area %*
1-Phenyldodecane	25.6	24.8	23.5	23.6	17.7	17.6
2-Phenyldodecane	17.1	17.2	18.0	17.7	16.3	16.4
3-Phenyldodecane	19.6	20.5	20.9	21.1	16.8	17.2
4-Phenyldodecane	19.4	17.7	18.5	17.2	16.9	15.4
5-Phenyldodecane	18.0	19.8	—	—	16.1 } 32.3	33.4
6-Phenyldodecane	—	—	19.1	20.4	16.2	

SUMMARY

A method is described for the gas chromatographic analysis of phenylalkanes. Retention times are listed at 240°, 270° and 320° for compounds from the phenylhexanes through the phenyleicosanes and for *n*-paraffin reference standards from *n*-decane to *n*-tetracosane.

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Short Communications

The nonlinearity of the signal response and the inversion effect in flame ionization detection

A further substantial development of gas chromatography has been achieved by means of highly sensitive detectors utilizing the ionization of gases and vapours. The flame ionization detector constitutes one of the important types[1,2] of these detectors. Up to now, attention has been mainly devoted to the aspects of construction and the attainment of maximal sensitivity[3,4]; in this stage, the detector has been used, primarily for qualitative analysis and for studying the separation features of the columns, etc.

Only recently have the working characteristics of the ionization flame detector been studied from the quantitative point of view[5-7]. Generally it has been stated that the relation between the magnitude of the signal and the concentration of the material in the carrier gas is linear[2-4]; recently, however, it has been reported that this linearity is roughly valid up to a concentration of 0.5 vol.% of the component in the carrier gas[5]. A negative deviation from linearity has been noticed at a concentration of 2% of the component[8].

Some observations made during our own experiments[7] with this detector are given in the following short communication.

The detector consisted of a steel capillary with an inner diameter of 0.5 mm and a wall thickness of 0.15 mm, above which two electrodes (made from 0.6 mm platinum wire) were placed across the center of the flame, perpendicular to its axis. The distance of the upper electrode from the burner was 10 and 15 mm, respectively. The distance of the lower electrode was variable. A voltage of 180 V was applied to the electrodes from an anode battery. Recording was made by means of a compensograph EZ-3, constructed by the National Plant "Laboratorní přístroje" (Laboratory Apparatuses), Prague (the input resistance was 500 kΩ and the scale 2 mV/280 mm). The flow rates of nitrogen as carrier gas and hydrogen as burning gas, fed directly into the inlet of the burner, are given in the legends to the figures.

It was found that in the case of low flow rates of the gases the inversion effect occurs at component concentrations above 0.6 vol.% (Fig. 1). The occurrence of nonlinearity of the detector signal response and the inversion of the chromatographic curves may be influenced by the flow rate, as well as by the position of the lower electrode. This effect is dependent on the form of the flame, however, especially on the form of the inner blue cone of the flame. The detector only functions correctly when the lower electrode is situated directly within the space where the oxidation of the

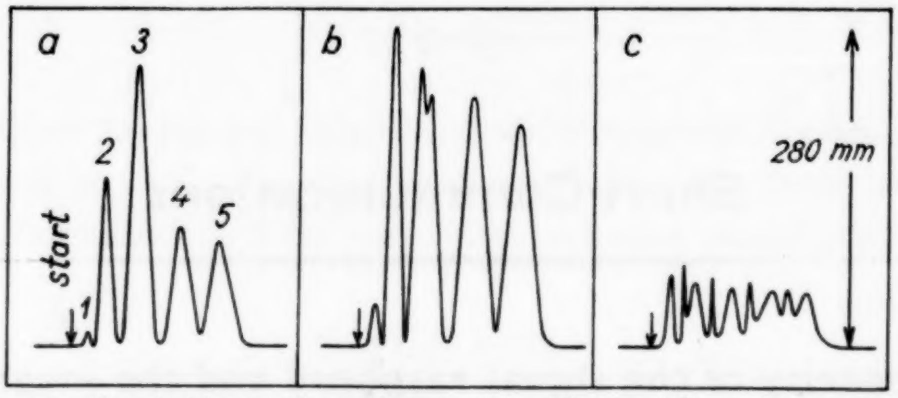

Fig. 1. Chromatograms of mixtures of methane (1), ethane (2), propane (3), isobutane (4) and *n*-butane (5). (a) sample volume 0.2 ml; reduction of the sensitivity of the compensograph 1 : 5; (b) sample volume 0.4 ml; reduction of the sensitivity of the compensograph 1 : 5; (c) sample volume 2.0 ml; reduction of the sensitivity of the compensograph 1 : 20. (45 g of 25% w/w Apiezon L on Sterchamol of 0.2–0.4 mm; temperature 90°; flow rate of nitrogen 0.3 ml/sec; additional hydrogen 0.25 ml/sec; distance of the lower electrode from the orifice of the burner 1.0 mm).

organic material begins, *i.e.*, when the burner serves as electrode[3–5] and/or when the lower special electrode is inserted into the inner cone of the flame[7]. Under our experimental conditions the optimal ratio of the carrier gas (nitrogen) and the additional gas (hydrogen) was 1 : 1–2.

The estimated inversion effects appeared when the lower electrode was placed across the flame 1.0 mm above the orifice of the burner; at the same time, the top of the inner cone of the flame extended above the level of the inserted electrode. The inversions occurred at a concentration of 0.65 vol. % of propane and butane, respectively, at flow rates of 0.3 ml N_2/sec and 0.25 ml H_2/sec. If the flow rate of the carrier gas is increased the inversion effect disappears.

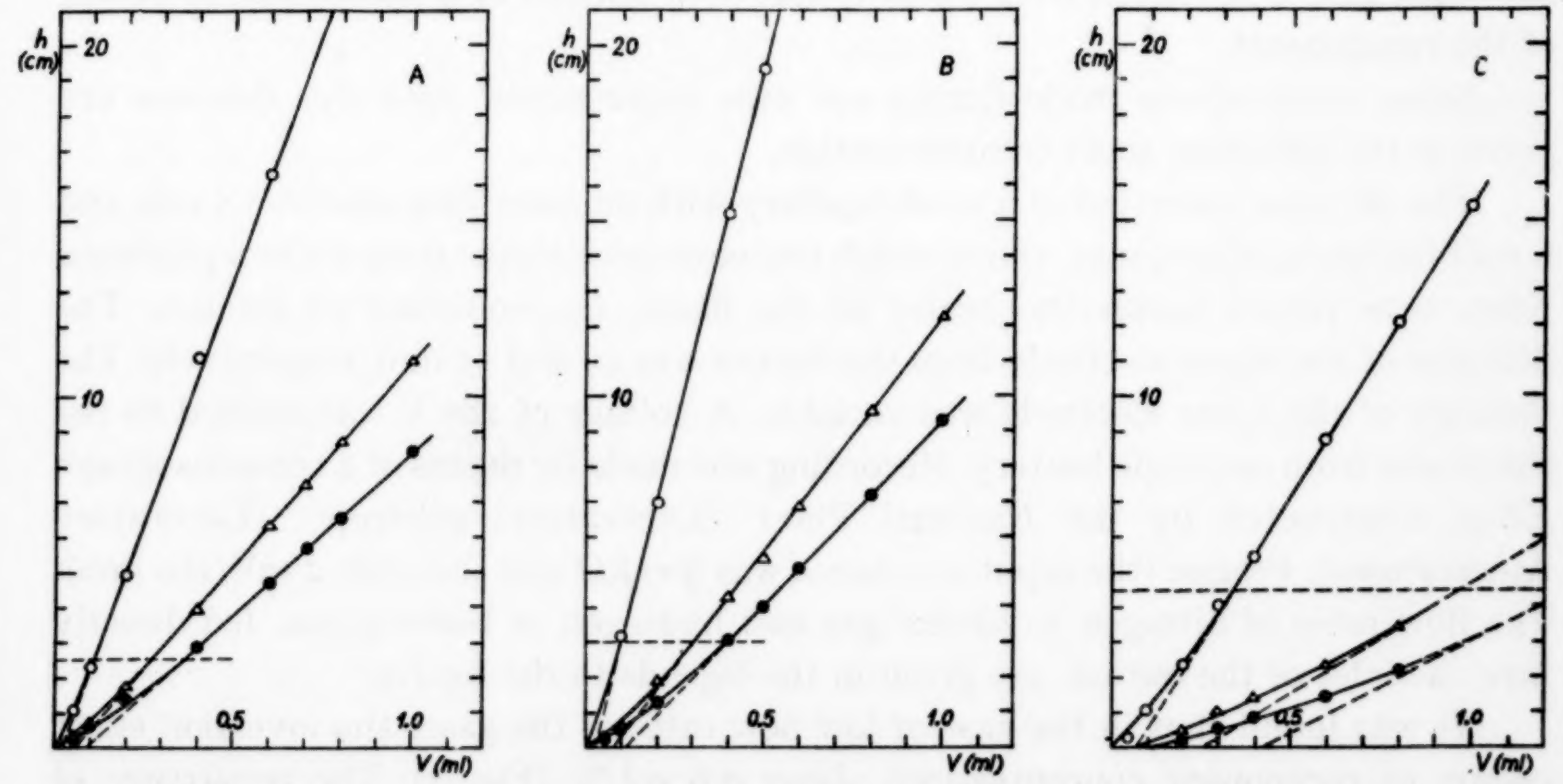

Fig. 2. Calibration curves for propane (o), isobutane (△) and *n*-butane (●) at distances of the lower electrode above the orifice of the burner of: (A) 0.5 mm, (B) 1.5 mm and (C) 3.0 mm. (9 g of 25% w/w squalane on Sterchamol of 0.2–0.4 mm; temperature 22°; flow rate of nitrogen 0.7 ml/sec and of hydrogen 0.6 ml/sec; distance between the two electrodes 10 mm; voltage applied to the electrodes 180V; 1.00 ml of diluted sample contained 0.0075 ml propane, 0.0032 ml isobutane and 0.0030 ml *n*-butane; the concentration of the components in the carrier gas at the peak maximum was in 1.00 ml of the diluted sample in vol. %: propane 0.086% isobutane 0.020% and *n*-butane 0.015%).

The position of the electrodes, especially that of the lower one, with respect to the direction of the gas stream through the burner influences the linearity of the signal also in the range of small concentrations (Fig. 2). While the position and form of the upper electrode had practically no effect, either on the sensitivity or on the linearity of the signal, at variable positions of the lower electrode above the orifice of the burner, deviations from linearity were observed even with small concentrations of the vapours analysed. In Fig. 2, calibration curves for the same concentrations of gases and for different positions of the lower electrode of 0.5 to 1.5 and 3.0 mm above the orifice of the burner may be seen. The range of the nonlinearity of the signal increases with the distance of the electrode from the burner.

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Ninhydrin as a reagent for N-methyl-amino acids

In the course of work reported elsewhere[1] a method was needed for estimating valine and N-methyl-leucine in solutions containing both amino acids. N-Methyl-amino acids normally react weakly on paper chromatograms sprayed with ninhydrin[2] and except for sarcosine[3] have not been observed to react in the test-tube[4,5]. Because the use of ninhydrin would permit the estimation of both amino acids on the same chromatogram, conditions were sought under which both primary- and N-methyl-amino acids would react to give spots of satisfactory intensity.

In the reaction of most primary amino acids with ninhydrin, optimal colour yields are obtained using a buffer of pH 5[3]. Under similar conditions both sarcosine and N-methyl-leucine reacted optimally at pH 6.5, at which pH leucine also gave a satisfactory, although not maximal, colour yield. Based on these observations, a technique has been developed for the detection and estimation of primary- and N-methyl-amino acids on paper chromatograms. The study was limited to the N-methyl derivatives of glycine, valine, leucine and isoleucine.

Chromatograms on Whatman No. 3 MM paper are run in the solvent system *tert.*-butanol–aqueous ammonia (sp.gr. 0.90)–water in the proportions 20:1:4. This solvent[6] gives good resolution of the aliphatic primary- and N-methyl-amino acids. The papers are dried in a current of warm air. For quantitative estimation the technique described by PEART[7] is employed. A 1% solution of ninhydrin in *n*-butanol previously equilibrated with 0.05 M phosphate buffer of pH 7 is used to spray the chromatograms, which are then heated at 105° for 30 min. Spots are excised and eluted, and the optical density at 570 mμ compared with that of standards run on the same paper.

For routine qualitative use the following reagent is also satisfactory.

Solution 1. 0.33% ninhydrin in *tert.*-butanol;

Solution 2. Glacial acetic acid–water–pyridine, 1:5:5.

Equal volumes of the two solutions are mixed just before use, the papers are sprayed lightly with the mixture and heated at 100–110° for 10–15 min.

Both reagents reveal primary- and N-methyl-amino acids as purple spots of comparable intensity, the limit of detection being about 0.01 μmole. The N-methyl-amino acids may readily be distinguished by the very weak colour they produce when sprayed with a solution of ninhydrin in water-saturated *n*-butanol containing 2% of glacial acetic acid.

It is a pleasure to thank Miss M. E. BROWN for technical assistance.

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Isomerization of terpenoid hydrocarbons by acid-washed alumina

Numerous reports have appeared[1] on the structural alteration of organic compounds during column chromatography on alumina or silicic acid. However, the properties of "acid-washed alumina" in this regard have not been investigated. The present

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* Mention of a specific commercial product does not constitute endorsement by the United States Department of Agriculture.

report concerns the isomerization of two terpenoid hydrocarbons, squalene and neophytadiene, during chromatography on this adsorbent.

Squalene (Eastman-Kodak*) was purified by fractional distillation, and the purified material was chromatographed (5 g) on a column (1.25 in. × 4.5 in.) of Merck acid-washed alumina (activated at 160° for 15 h). The column was washed with petroleum ether which completely eluted the terpene in seven successive fractions of 100 ml each. The infrared spectra of the seven fractions were identical: the broad band at 11.7–12.4 μ ($R_1R_2C{=}CHR_3$) of squalene had disappeared, a moderate band at 11.25 μ ($R_1R_2C{=}CH_2$) had appeared and the absorption at 6.01 μ ($R_1R_2C{=}CHR_3$) of squalene had shifted to 6.07 μ ($R_1R_2C = CH_2$). These spectral shifts and the presence of a slight inflection at 234 mμ[2] were suggestive of a squalene → regenerated squalene[3] isomerization during chromatography. However, minor infrared spectral differences were observed between the eluted material and regenerated squalene: the former showed weaker, less distinct bands in the 8.5–10.2 μ region, a slight inflection at 7.35 μ (isopropyl-) and no adsorption at 11.7–12.4 μ. Thus, the possible presence of isomers other than regenerated squalene in the eluted material was indicated.

Isomerization of neophytadiene[4–6] (3-methylene-7,11,15-trimethyl-1-hexadecene) also occurred under the above conditions. The infrared spectrum of the eluted material showed an intense band at 10.38 μ (*trans* —CH=CH—) in place of the 10.1, 11.1 and 11.2 μ bands ($R_1R_2C{=}CH_2$ and $RCH{=}CH_2$) of neophytadiene; these characteristics are similar to the spectra reported[4,7] for Phytadiene C and Phytadiene Fraction V.

In studies on the isolation of natural products, the possibility of isomerization of terpenes during chromatography on acid-washed alumina should be recognized. Perhaps the reported presence[2,8] of regenerated squalene in cigarette smoke condensates was the result of such isomerization.

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A new paper chromatographic technique for the separation of cholesterol and cholesteryl esters

In our previous papers[1-4] we described a method for the qualitative and quantitative separation of cholesterol and cholesteryl esters by paper chromatography. During recent years several authors have published other methods[5-9]. In our present study a new modification of the reversed phase partition system was used for the identification of these substances.

Experimental

Cholesteryl esters. Cholesteryl formate, acetate, propionate, caproate, caprylate, laurate and myristate were prepared in our laboratory by the method of PAGE AND RUDY[10] or that of SWELL AND TREADWELL[11] with minor modifications. Cholesteryl palmitate, stearate, oleate and linoleate were obtained from Merck Ltd. The iodine values of these substances are given in Table I.

TABLE I

IODINE VALUES OF CHOLESTERYL ESTERS

Ester	*Found*	*Literature*[10]
Cholesteryl formate	59.9	60.8
Cholesteryl acetate	58.8	59.8
Cholesteryl propionate	57.6	57.3
Cholesteryl caproate	52.0	52.4
Cholesteryl caprylate	48.5	49.1
Cholesteryl laurate	44.2	44.4
Cholesteryl myristate	41.6	42.4
Cholesteryl palmitate	39.8	40.6
Cholesteryl stearate	39.4	39.3
Cholesteryl oleate	74.3	73.9
Cholesteryl linoleate	115.5	114.6

Solvents. Acetic acid, p.a., chloroform, p.a., paraffin oil (paraffinum liquidum) sp.gr. 0.880. Other reagents were ordinary laboratory chemicals of reagent grade and were used without further purification.

Impregnation of paper. Whatman No. 3 filter paper (15 × 30 cm) was drawn through a 0.5% (v/v) solution of paraffin oil in ether or other solvents (*e.g.* chloroform, benzene). After a few minutes the solvents had evaporated and the strips were ready for chromatography. They could be kept for more than one month without any loss of their "activity". The cholesteryl esters (50–100 μg of each substance) were applied as a narrow line (2 cm long) on the start, 2 cm from the lower end of the paper.

Mobile phases. System A: acetic acid saturated with paraffin oil (after equilibration the excess of paraffin oil was filtered off); ascending chromatography for 5–6 h. *System B*: acetic acid–chloroform–paraffin oil, 80:15:5 (v/v/v); ascending chromatography for 3–4 h.

All chromatographic runs were carried out at room temperature (20 ± 2°).

Detection. The chromatograms were dried at 80–90° or at room temperature. Saturated esters together with unsaturated esters were detected by spraying with phosphotungstic acid (10% in ethanol) or antimony trichloride (50% in acetic acid).

Unsaturated esters were identified specifically as dark brown spots, by staining with a 1% solution of potassium permanganate. The excess of $KMnO_4$ was immediately removed in running water. In several cases iodine vapours or a 1% solution of iodine in methanol were also used.

In Table II the R_F values of cholesteryl esters are summarized. In Figs. 1 and 2 typical chromatograms of cholesteryl esters are given.

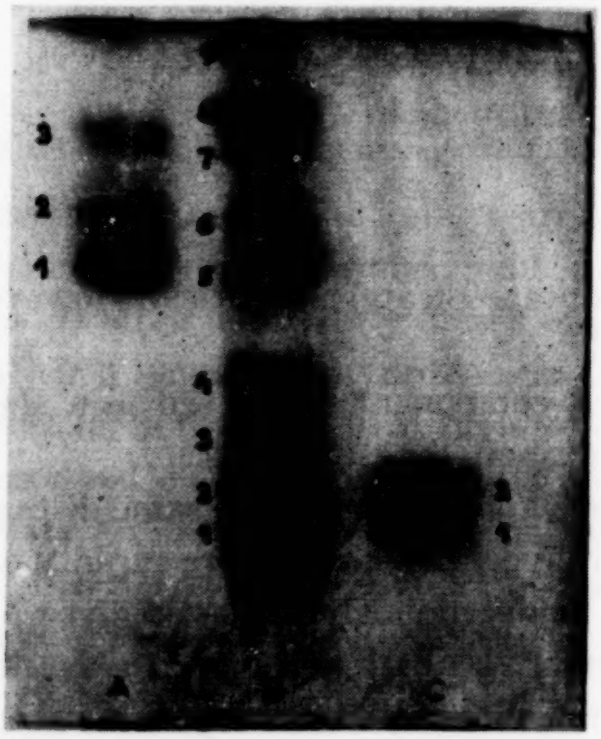

Fig. 1. System B. A: (1) cholesteryl caprylate; (2) cholesteryl caproate; (3) cholesteryl propionate. B: (1) cholesteryl stearate; (2) cholesteryl palmitate; (3) cholesteryl myristate; (4) cholesteryl laurate; (5) cholesteryl caprylate; (6) cholesteryl caproate; (7) cholesteryl propionate; (8) cholesteryl acetate and formate; (9) cholesterol. C: (1) cholesteryl stearate; (2) cholesteryl palmitate.

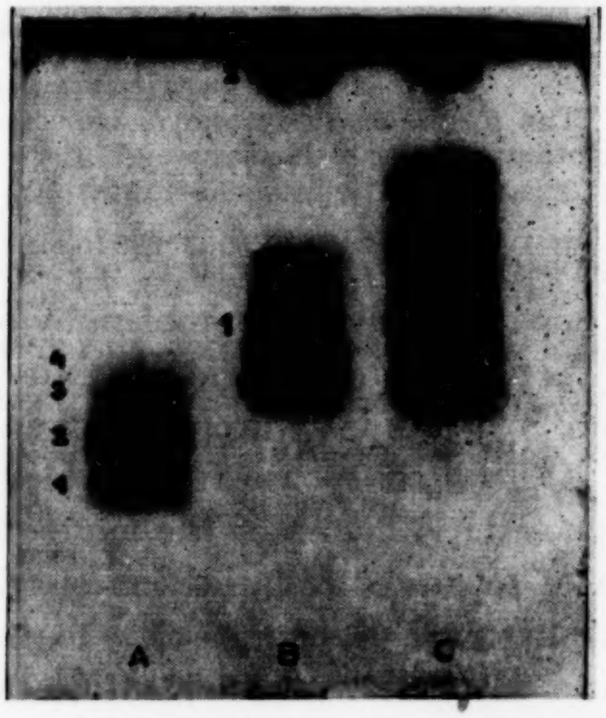

Fig. 2. System B. A: Unsaturated cholesteryl esters of human blood serum isolated on an aluminium oxide column. (1) cholesteryl oleate; (2) cholesteryl linoleate; (3) cholesteryl arachidonate; (4) cholesteryl linolenate (?). B: Unsaturated triglycerides of human blood serum. (1) triglycerides; (2) mono- and diglycerides and cholesterol and free fatty acids. C: Mixture of triglycerides of olive and linseed oil.

Discussion and results

The present modification is a very useful technique for the separation of many cholesteryl esters. The lowering of the amount of the stationary phase and a minor modification of the mobile phases, give rise to greater differences in mobilities of the individual esters. Also the time necessary for complete resolution is shorter than in the method using a 5% or 10% solution of paraffin oil for impregnation of the paper. Satisfactory separations are obtained without prior equilibration of the chromatograms. On the other hand, resolution of a mixture of saturated and unsaturated compounds cannot be accomplished satisfactorily with these solvent systems alone.

TABLE II

R_F VALUES OF CHOLESTERYL ESTERS

Compound	R_F *System B**
Cholesterol	0.96
Cholesteryl formate	0.87
Cholesteryl acetate	0.87
Cholesteryl propionate	0.81
Cholesteryl caproate	0.72
Cholesteryl caprylate	0.65
Cholesteryl laurate	0.52
Cholesteryl myristate	0.45
Cholesteryl palmitate	0.37
Cholesteryl stearate	0.30
Cholesteryl oleate	0.38
Cholesteryl linoleate	0.46

* The R_F values in system A are somewhat lower and this system is more suitable for the resolution of lower molecular weight derivatives (C_2–C_8).

The position of "critical pairs" (*e.g.* cholesteryl palmitate–oleate) is identical. However, we hope to resolve this problem by other methods.

Separation is best if about 50–70 μg of each component is spotted in a short band. Spotting as a "drop" is not so good and the spots tend to streak.

Other lipids such as triglycerides (triolein) move approximately the same distance as cholesteryl linoleate. The more unsaturated triglycerides move more rapidly and can be separated from higher esters. Free fatty acids, monoglycerides, diglycerides and phospholipids migrate close to the solvent front. In general, however, triglycerides do not interfere with the identification of cholesteryl esters, since they do not react with phosphotungstic acid and antimony trichloride or in other tests. For the specific detection of unsaturated esters, it is necessary to isolate the whole ester fraction by column chromatography on aluminium oxide and then separate the individual components.

Quantitative estimation of unsaturated esters was carried out by direct densitometry. The coloration with phosphotungstic acid or antimony trichloride is not quite stable and it is still not suitable for quantitative measurements in this arrangement.

The systems described here have been successfully applied to the separation of higher cholesteryl esters in various biological materials. In addition, these systems are also very useful for the identification of individual triglycerides.

Further results in this field will be reported in future papers.

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Qualitative separation of choline esters by means of high voltage paper electrophoresis

Choline esters occur in living material and these compounds as well as several synthetic choline esters are of considerable pharmacological interest. Separation and identification is often effected by means of paper chromatography. Since this procedure takes rather a long time, hydrolysis of the esters may easily occur. Therefore the authors have for some time used high voltage paper electrophoresis. The method has been described earlier by one of the authors in connection with studies on thiocholine esters[1]. About 20 choline esters and γ-aminobutyric acid have now been investigated as regards their behaviour in various buffer solutions, various staining methods having been used.

Experimental

High voltage paper electrophoresis. The esters were dissolved in distilled water and applied with a micropipette. Paper electrophoresis was carried out as described earlier[1], usually at a voltage of 1,500 V, and 30 V/cm. Thirty to sixty minutes were sufficient for the electrophoresis.The temperature of the paper was 12–13° during electrophoresis

Buffer solutions. The following buffer systems were applied:

Triethylamine–CO_2, 0.05 *M*, pH 9.1.
Acetate–acetic acid, 0.20 *M*, pH 5.0.
Acetate–acetic acid, 0.20 *M*, pH 4.6.
Boric acid–sodium hydroxide, 0.10 *M*, pH 10.0.

Identification. After electrophoresis the papers were air-dried. For detection of the spots the following methods were used: Quaternary ammonium was identified with dipicrylamine[2] or with phosphomolybdic acid[3]. In some cases iron-hydroxamate[4] was used. γ-Aminobutyric acid was detected with ninhydrin.

Results

Table I shows the mobilities relative to choline of a number of choline esters in various buffer systems. Separations of mixtures of acetylcholine, propionylcholine and butyrylcholine and of γ-aminobutyric acid, γ-aminobutyrylcholine, acetylcholine and choline were carried out (see Fig. 1). γ-Aminobutyric acid showed tailing. The reason for this is probably dissociation of the acid. The relatively small difference between the mobility of γ-aminobutyric acid at pH 4.6 and that at pH 10.0 may be explained by the values of the dissociation constants of the acid[5]; at 10° pK_1 (—COOH) was found to be 4.05 and pK_2 (—NH_3^+) was 11.02.

The amount of choline esters most suitable for detection with dipicrylamine or phosphomolybdic acid is 1–10 γ per spot. Amounts down to 0.5 γ could be detected in the case of a few substances, *e.g.* succinylcholine.

TABLE I

MOBILITIES OF SOME CHOLINE ESTERS IN FOUR DIFFERENT BUFFER SOLUTIONS

Migration rates are relative to the rate of choline chloride or choline iodide.

Compound	*Buffer solutions*			
	Acetate *pH = 4.6*	*Acetate* *pH = 5.0*	*TEA* *pH = 9.1*	*Borate* *pH = 10.0*
Acetylcholine iodide	0.89 ± 0.05	0.91 ± 0.04	0.89 ± 0.01	0.96 ± 0.03
Propionylcholine iodide	0.79 ± 0.03	0.87 ± 0.01	0.84 ± 0.01	0.89 ± 0.02
Butyrylcholine iodide	0.70 ± 0.02	0.80 ± 0.01	0.83 ± 0.01	0.82
n-Valerylcholine iodide	0.69 ± 0.04	0.70 ± 0.05	0.72 ± 0.06	0.80 ± 0.01
Isovalerylcholine iodide	0.69 ± 0.03	—	0.74 ± 0.06	0.79 ± 0.02
Acetyl-α-methylcholine iodide	0.81 ± 0.03	0.85 ± 0.02	0.83 ± 0.02	0.90 ± 0.02
Acetyl-β-methylcholine iodide	0.93 ± 0.01	0.91	0.89 ± 0.01	—
Acetyl-β,β-dimethylcholine iodide	0.69 ± 0.05	0.71 ± 0.01	0.76 ± 0.02	0.86 ± 0.02
Acrylylcholine iodide	0.80 ± 0.01	0.79 ± 0.01	0.85 ± 0.03	0.88 ± 0.03
β,β-Dimethylacrylylcholine iodide	0.64 ± 0.01	0.64 ± 0.01	0.69 ± 0.05	0.79 ± 0.01
α-Methylcrotonylcholine iodide	0.70 ± 0.02	—	0.69 ± 0.04	0.81 ± 0.04
Succinylcholine chloride	1.02 ± 0.05	1.03 ± 0.02	1.01 ± 0.03	1.08 ± 0.01
Phenylsuccinylcholine iodide	0.91 ± 0.04	0.84 ± 0.03	0.85 ± 0.03	0.95 ± 0.03
Benzoylcholine chloride	0.68 ± 0.02	0.66 ± 0.04	0.69 ± 0.03	0.73 ± 0.02
Nicotinylcholine iodide	0.66 ± 0.02	0.62 ± 0.04	0.70 ± 0.05	0.74 ± 0.02
Myristylcholine iodide	starting point	starting point	starting point	starting point
β-Bromethyltrimethylammonium bromide	0.96 ± 0.02	0.88 ± 0.01	0.93 ± 0.03	0.97 ± 0.02
β-Methylcholine iodide	0.91	—	—	0.96 ± 0.02
γ-Aminobutyrylcholine iodide	1.20 ± 0.04	1.14 ± 0.12	1.08	1.14
γ-Aminobutyric acid	0.30 ± 0.10	—	0.12 ± 0.04	0.06 ± 0.08

The electrophoretograms show that no hydrolysis occurs during the time necessary for electrophoresis. Since there is no great difference in the mobility of the choline esters in the different buffer systems used, the authors recommend buffers of pH 4–5 for electrophoresis. It has been shown, for instance by LARSSON[6], that the hydrolysis of choline esters is slowest at pH around 4. It has furthermore been observed that spots are easier to detect with buffers of lower pH, especially if dipicrylamine is used for staining, because the background, the sprayed paper, is less coloured than at higher pH.

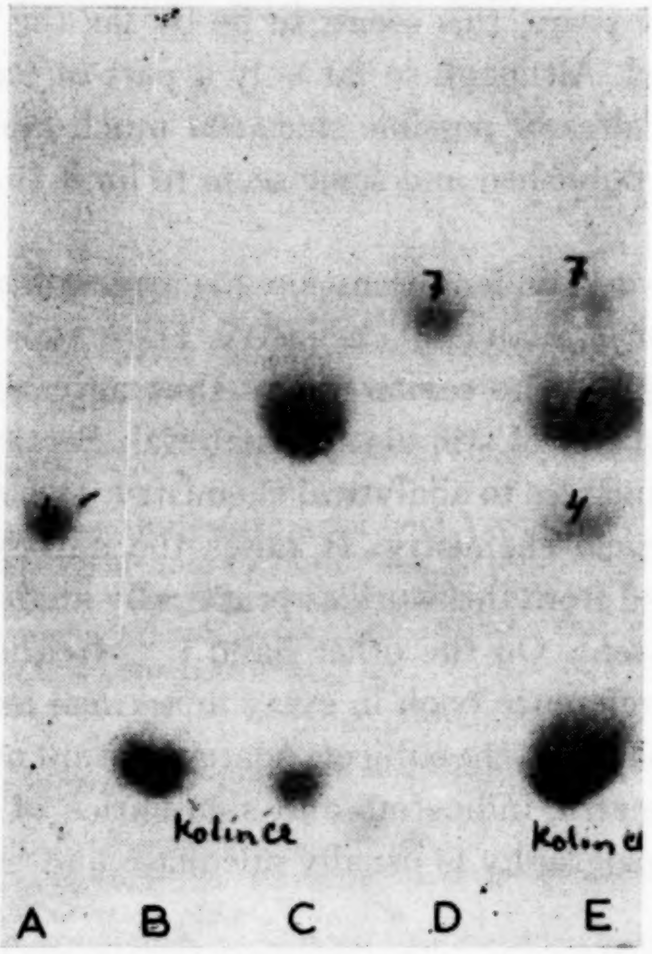

Fig. 1. High voltage paper electrophoresis of a mixture of acetylcholine iodide (4), propionylcholine iodide (6), butyrylcholine iodide (7) and choline chloride at pH 4.6. A, B and D are pure substances, C and E mixtures.

It was found that different esters show a somewhat different colour when sprayed with dipicrylamine. The spots of acetylcholine and choline were blue-red while the spots of the other esters were orange-red. It has also been observed that phosphomolybdic acid is a more reliable developer than dipicrylamine, but dipicrylamine is somewhat more sensitive. The iron-hydroxamate developer sometimes failed to give a coloured spot.

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BOOK REVIEW

Treatise on Analytical Chemistry, a comprehensive account in three parts. Edited by I. M. KOLTHOFF AND F. J. ELVING with the assistance of E. B. SANDELL, published by Interscience Encyclopedia, Inc., New York, Part I, Theory and Practice, Volume 1, 1959, 810 pages, price single volume $ 17.50, subscription $ 15.00.

Of the numerous comprehensive treatises of analytical chemistry which have been launched in the last few years, this seems to be by far the best as far as plan and conception are concerned. Although so far only a part of Part I, Volume 1, has appeared a comparison is already possible since not much more of the other comprehensive works has been published and some seem to limit themselves to this original burst of effort.

Section A of the work under discussion (93 pages) deals with the objectives, functions and limitations of analytical chemistry. These topics would encourage most authors to emit platitudes. The reviewer was thus agreeably surprised to find all chapters fascinating and full of stimulating material. Section B deals with the application of chemical principles to analytical chemistry. It is really a concise and comprehensive text of physical chemistry. It raises the question whether any part of chemistry can be omitted from this work as practically all fields of chemistry concern the analyst to some extent. On the other hand the treatise of physical chemistry will be invaluable as a reference book in every analytical laboratory and is more up to date than most text books on the subject. A large amount of data, such as instability constants of complexometric indicators and solubilities of salts etc. are presented in the text and the bibliography is usually adequate and contains numerous recent references.

The editors of the book are to be congratulated for their gift of organisation as well as for the uniformly high scientific standard of the contributions.

ÜBERSICHT

KONTINUIERLICHE ELEKTROPHORESE UND ZWEIDIMENSIONALE ELEKTROCHROMATOGRAPHIE*

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(Eingegangen den 9. Januar 1960)

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* Zum zehnten Jahrestag der kontinuierlichen Elektrophorese.

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I. EINLEITUNG

Das Prinzip der kontinuierlichen elektrophoretischen Trennung beruht darauf, dass man quer zu einem Flüssigkeitsstrom ein gleichgerichtetes elektrisches Feld überlagert, und auf einer bestimmten Stelle die zu trennende Mischung kontinuierlich zuströmen lässt. Es ist prinzipiell gleichgültig, ob sich der Flüssigkeitsstrom frei oder durch ein poröses Adsorbens bewegt. Die elektrisch geladenen Teilchen wandern in der Richtung der Resultante, bzw. werden je nach den elektrophoretischen Beweglichkeiten aus der Richtung des Flüssigkeitsstromes mehr oher weniger stark abgelenkt, und können getrennt am Ende ihrer Bahnen kontinuierlich aufgefangen werden.

Falls sich die Flüssigkeitsströmung mit einem quer zu ihr überlagerten gleichgerichteten elektrischen Feld durch ein poröses Adsorbens bewegt, und die zu trennende Mischung diskontinuierlich auf einer geeigneten Stelle aufgetragen wird, wandern die Komponenten des zu trennenden Gemisches in Zonen, deren Geschwindigkeiten und Richtungen die vektorische Summe von zwei quer zueinander stehenden Geschwindigkeiten sind: (a) in der Richtung des elektrischen Feldes aus der elektrochromatographischen Geschwindigkeit, und (b) in der Richtung der Flüssigkeitsströmung aus der chromatographischen Geschwindigkeit. Dadurch werden die Zonen der Komponenten in einer Ebene örtlich aufgetrennt, und es resultiert eine zweidimensionale elektrochromatographische Trennung.

Die kontinuierliche elektrophoretische Trennung sowie auch die zweidimensionale elektrochromatographische Trennung unterliegen, obwohl sie sich durch die Resultate der Trennung grundsätzlich voneinander unterscheiden, offensichtlich gleichen Gesetzen, und können mittels derselben Apparaturen ausgeführt werden, wenn die Apparatur in einem porösen Trägersystem arbeitet, das sich gegenüber der zu trennenden Komponenten als Adsorbens auswirken kann.

Kontinuierliche Elektrophorese an einem adsorptiven porösen Trägersystem unterscheidet sich grundsätzlich von allen bekannten diskontinuierlichen eindimensionalen und zweidimensionalen Trennungen, ohne Rücksicht darauf, ob es sich um chromatographische Trennungen, Trennungen durch Ionenaustausch, elektrochromatographische Trennungen, oder kontinuierliche chromatographische Trennungen handelt, indem eine langsamer wandernde Zone nicht von den schneller wandernden Zonen wegen teilweiser und reversibler Adsorption am porösen Träger verunreinigt wird. Durch kontinuierliche Elektrophorese getrennt wandert jede Komponente auf ihrer eigenen Bahn. Somit ist eine Möglichkeit gegeben, ohne besonderen Aufwand durch kontinuierliche Elektrophorese absolute Trennungen zu erreichen. Dieses ist von ausschlaggebender Bedeutung für die Trennung radioaktiver Ionen mit hoher spezifischer Aktivität, da hier auch die kleinste Verunreinigung grosse Fehler verursachen kann. Da die Beweglichkeit einfacher anorganischer Ionen im elektrischen Feld in grossen Konzentrationsbereichen individuellen Gesetzen unter-liegt, ist es möglich, unter Verwendung flüchtiger oder verbrennbarer Grundelektrolyte, durch kontinuierliche elektrophoretische Trennung, absolut reine Radionuklide bzw. Radionuklide ohne Träger (carrier free) zu erhalten.

Durch diese Methode können prinzipiell auch qualitative und quantitative Analysen eines Gemisches ausgeführt werden, obwohl die kontinuierliche Elektrophorese primär nur als eine Methode für präparative Trennungen angesehen wird. Besonders im Gebiet der Trennungen von Radionukliden wird sich die kontinuierliche Elektrophorese als eine neue und sehr praktische analytische Methode einführen können. Man braucht nur für eine bestimmte Zeit einen neuen Satz von Auffanggläsern in die Apparatur einzulegen, und zwar im Augenblick, wenn einmal der stationäre Zustand des kontinuierlichen Prozesses erreicht ist, d.h. wenn alle Radionuklide die Apparatur mit derselben Geschwindigkeit verlassen, mit der sie in die Apparatur eingeführt werden. Eine qualitative und quantitative radiometrische Analyse der Radionuklide aus den Auffanggläsern kann dann in üblicher Weise ausgeführt werden, falls die Zufuhr der zu trennenden Substanzmischung durch eine geeignete Dosierpumpe mit konstanter und bekannter Geschwindigkeit erfolgt. Ein weiterer Vorteil der kontinuierlichen Elektrophorese im Gebiet der Trennungen von Radionukliden besteht in der Möglichkeit einer relativ einfachen Konstruktion von Fernsteuerung, bzw. einer vollautomatischen Steuerung.

Die meisten bisher über kontinuierliche elektrophoretische Trennungen veröffentlichten Arbeiten gehören jedoch zu den für die Elektrophorese "klassischen" Gebieten: der klinischen Biochemie, der physiologischen Chemie, der Biochemie, der Pharmazie und der organischen Chemie. Es wurden erfolgreich Trennungen von Serumproteinen und anderen proteinhaltigen Gemischen, Proteinhormonen, Toxinen und Antikörpern, Fermenten, Aminosäuren und Peptiden, Zuckern, antibiotischen Substanzen, Viren, Alkaloiden, Gerbstoffen, natürlichen und künstlichen Farbstoffen u.s.w. durchgeführt.

Heutzutage ist die kontinuierliche Elektrophorese mehr oder weniger noch auf Trennungen im Laboratoriumsmasstab beschränkt. Diese Beschränkung ist aber keinesfalls prinzipieller Natur. Für die Ausführung in grösserem Masstab fehlt nur noch eine entsprechende technische Lösung, obwohl in dieser Richtung mehrere Wege bereits eingeschlagen sind.

Zum ersten Male wurde das Prinzip der zweidimensionalen elektrochromatographischen Trennung im Jahre 1948 von HAUGAARD UND KRONER[1] angegeben, und dann wurde die kontinuierliche elektrophoretische Trennung von GRASSMANN[2,3], sowie von SVENSSON UND I. BRATTSTEN[4] ungefähr gleichzeitig im Jahre 1949 veröffentlicht. Für die kontinuierliche Trennung dienten als Trennkammern flache Küvetten, die mit Glaspulver oder Seesand gefüllt waren. Darauf folgten Arbeiten über die mit wesentlich geringerem experimentellen Aufwand erreichbaren kontinuierlichen Trennungen am Filtrierpapier von GRASSMANN UND HANNIG[5] (1950), und (1951) von DURRUM[6] und STRAIN UND SULLIVAN[7]. Seitdem sind mehrere Referate[8-13] und Monographien [14-22] über Elektrophorese erschienen, die auch, mehr oder weniger, die kontinuierliche Trennung behandeln.

2. APPARATUR

Die Anforderungen an eine einwandfrei arbeitende Apparatur für kontinuierliche Elektrophorese sind ziemlich anspruchsvoll. Obwohl bereits einige einfache Ausführungen, die man sich selbstätig im Laboratorium anfertigen kann, in der Literatur[46, 47] vorliegen, wird man im allgemeinen auf eine der im Handel erhältlichen Ausführungen angewiesen sein. Diese Apparate sind in der Regel von den Herstellerfirmen nach in der Literatur beschriebenen Prototypen und entsprechenden Laboratoriumserfahrungen gebaut und weiterentwickelt worden.

Für die Schärfe des Trennprozesses ist beim kontinuierlichen Arbeitsverfahren die Zeitkonstanz der Geschwindigkeitsvektoren des Grundelektrolyten, der elektrischen Feldstärke und pH für jeden einzelnen Punkt des Trennraumes absolut unentbehrlich, trotz der durch den Stromtransport entwickelten Jouleschen Wärme. Die Konstanz der angegebenen Grössen von Punkt zu Punkt des Trennraumes ist aber im Gegenteil nicht absolut notwendig. Eine gut konstruierte Apparatur, in der die Trennung auf dem aufgespannten Bogen eines geeigneten Filtrierpapiers in einer feuchten Kammer erfolgt, wird den angegebenen Anforderungen genügen. Wegen relativ einfacher Bauart und einfacher Handhabung sind z. Z. solche Apparate am meisten verbreitet, und haben umfangreichste Anwendung für Trennungen im Laboratoriumsmasstab bereits gefunden. Bis vor Kurzem wurden ausschliesslich nur solche Apparate, die in feuchter Kammer arbeiten, serienmässig hergestellt und in den Handel gebracht.

Das kontinuierliche Trennverfahren in mit geeigneten Trägersubstanzen gefüllten küvettenförmigen Trennkammern bietet einige Vorteile gegenüber dem Verfahren auf Filtrierpapier oder Filtrierkartons, indem die vertikale Strömungsgeschwindigkeit des Grundelektrolyten in weiten Grenzen, je nach Bedarf der Trennung, variiert werden kann. Ausserdem wird die Verdunstung des Grundelektrolyten, die bei der Elektrophorese in feuchter Kammer zu bedeutenden Störungen führen kann, vollkommen verhindert, die Temperatur, auch bei grösseren Stromleistungen, durch Wasserkühlung nieder und annähernd konstant gehalten, und der Mengendurchsatz erheblich gesteigert. Die zu trennenden Substanzen kommen während des Trennprozesses nicht mit der Luft in Berührung. Derartige Apparaturen sind aber wesentlich komplizierter und kostspieliger im Aufbau, und obwohl sie zeitlich den Apparaten mit Filtrierpapiervorhang vorausstehen, wurden sie bis heute noch nicht in dem Masse vervollkommnet und entwickelt, dass sich ihre allgemeine Anwendung und Ausbreitung rechtfertigen würde.

In letzter Zeit wurde auch die freie kontinuierliche Elektrophorese, d. i. kontinuierliche Elektrophorese ohne kapillares Trägersystem, von mehreren Seiten aus angestrebt. In den U.S.A. wurde von einer Seite her[62] die kontinuierliche Elektrophorese-Konvektionsmethode vorgeschlagen, die nach dem Elektrodekantationsprinzip arbeitet, aber den grossen Nachteil hat, dass nur eine der Komponenten in einem Experiment rein zu gewinnen ist, die Methode ausserdem nur bei hochmolekularen Stoffen angewendet werden kann. Anderseits wurde eine auf dem Prinzip einer in einem Verdickungsmittel aufwärts gerichteten forcierten Laminarströmung beruhende Methode vorgeschlagen[57,58]. Der Vorteil der beiden Methoden besteht in der Möglichkeit, den Mengendurchsatz so stark zu steigern, dass angenommen werden kann, dass der Weg der kontinuierlichen Elektrophorese zum grosstechnischen Betrieb bereits eingeschlagen ist. Inzwischen wurde in Deutschland eine freie präparative Durchfluss-Elektrophorese-Anlage[60] für Arbeiten im Laboratoriumsmasstab entwickelt, und unter den Namen "Pheroplan" in den Handel gebracht. Ein wesentlicher Vorteil der letztgenannten Apparatur gegenüber den Apparaten mit Filtrierpapiervorhang liegt in der Möglichkeit, auch stark adsorbierbare Substanzen kontinuierlich zu trennen.

(*a*) *Apparate mit Filtrierpapiervorhang*

1. *Trennbogen in feuchter Kammer* (*System mit Verdunstung*)

(i) Apparate, die nach dem Vorbild der Anordnung von Durrum entworfen wurden. Fig. 1[23] gibt schematisch die Konstruktion der Trennapparatur nach Durrum[6] wieder. Sie ist zugleich die denkbar einfachste Anordnung für kontinuierliche Elektrophorese. In einer feuchten Kammer (in Fig. 1 nicht wiedergegeben) ist ein Filtrierpapierblatt frei über einer Pufferwanne aufgehängt. In der Pufferwanne wird ein konstantes Niveau des Grundelektrolyten aufrechterhalten. Die Lösung der zu trennenden Substanz befindet sich in einem kleinen Glasbehälter über der Pufferwanne; sie wird mittels eines Dochtes dem freihängenden Filtrierpapiervorhang zugeführt, und zwar auf einer geeigneten Stelle längs der Pufferwanne. Der Filtrierpapiervorhang ist am unteren Rande in Zacken zugeschnitten, die das Abtropfen des Grundelektrolyten in die Auffanggläschen ermöglichen. Die zwei breiter zugeschnittenen unteren Enden des Trennbogens tauchen in die Elektrodenflüssigkeit ein. Die Platinelektroden werden an eine Gleichstromquelle angeschlossen.

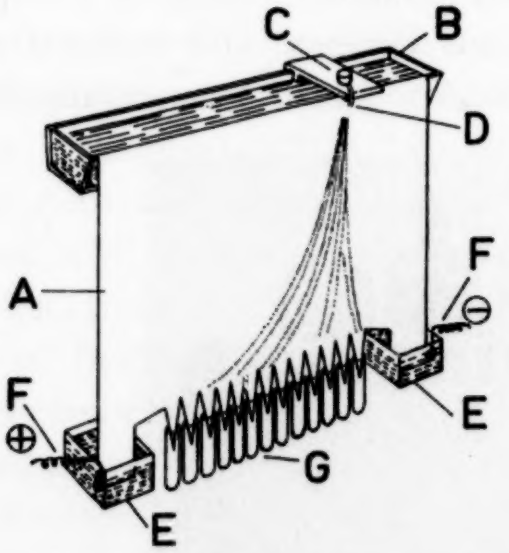


Fig. 1. Schematische Wiedergabe der Trennanlage nach Durrum[23]. A = Filtrierpapierbogen; B = Pufferwanne; C = Trennsubstanz; D = Papierdocht; E = Elektrodengefässe; F = Platinelektroden; G = Auffanggläschen.

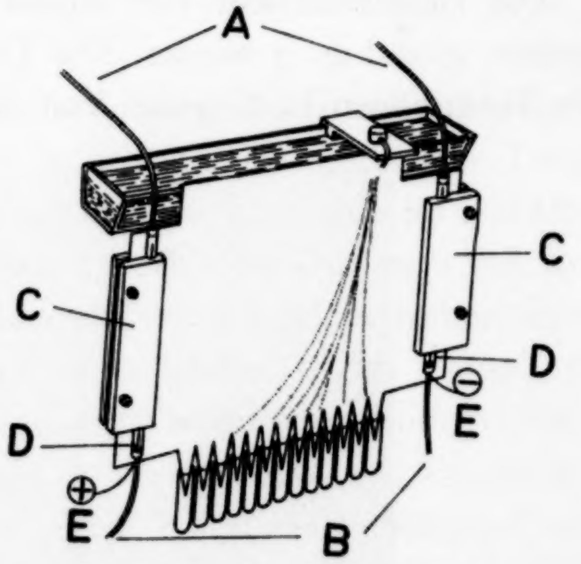


Fig. 2. Schematische Wiedergabe der vertikalen Flüssigkeitselektroden[23]. A = Einlauf des Puffers; B = Ablauf des Puffers; C = Halter aus Perspex; D = Cellophanschlauch; E = Stromanschluss an die Platindrahtelektroden.

Die beschriebene Apparatur ist konstruktiv sehr einfach, aber sie hat den Nachteil, dass die elektrische Feldstärke in der Richtung von unten nach oben am Filtrierpapier sehr rasch abfällt, was zu einer starken Krümmung der Wanderungswege führt. Diesen Nachteil versuchten DICASTRO UND SAN MARCO[24,25] durch je drei Paare aus den Trennbogen seitlich herausgeschnittener und übereinanderliegender Zipfel, die in eine Reihe übereinanderliegender Elektrodengefässe eintauchen, zu beseitigen. BIGHI UND TRABANELLI[26] haben der Grundausführung nach DURRUM ebenfalls noch ein Paar weitere höher stehende Elektrodengefässe zugegeben. Die Zipfel für die zusätzlichen Elektrodengefässe haben einen kleineren Abstand als die unteren Zipfel, und sind nicht am Rande des Trennbogens angebracht, sondern aus den Trennbogen durch einen U-förmigen Schnitt mehr gegen die Mitte gebildet. Dieses bewirkt eine grössere elektrische Feldstärke im oberen Teil des Trennbogens, wo sonst auch die vertikale Geschwindigkeit des Grundelektrolyten wegen Verdunstung grösser ist als unten. Diese Anordnung führt zu einer wirkungsvollen Rektifikation der Wanderungswege.

Die Firma Shandon, London, hat für ihren Apparat neben der aus Fig. 1 ersichtlichen Arbeitsweise auch alternativ vertikale Durchflusselektroden (Fig. 2[23]) vorgesehen, die kontinuierlich von zwei zusätzlichen Elektrolytbehältern gespült werden. Durch diese Massnahme wird die Verteilung des elektrischen Stromes und des Stromtransportes am Trennbogen weitgehend verbessert, und die Produkte der Elektrolyse aus der unmittelbaren Umgebung der Elektroden kontinuierlich abgeführt. In diesem Falle werden die Elektrodengefässe nicht benützt.

Die Gesamtansicht der Apparatur der Firma Shandon Scientific Company, London, gibt Fig. 3 wieder. Die Trennanlage ist aus Perspex, und so konstruiert, dass Trennbogen in Grössen von *ca.* 25 × 34 bis 46 × 57 cm Verwendung finden

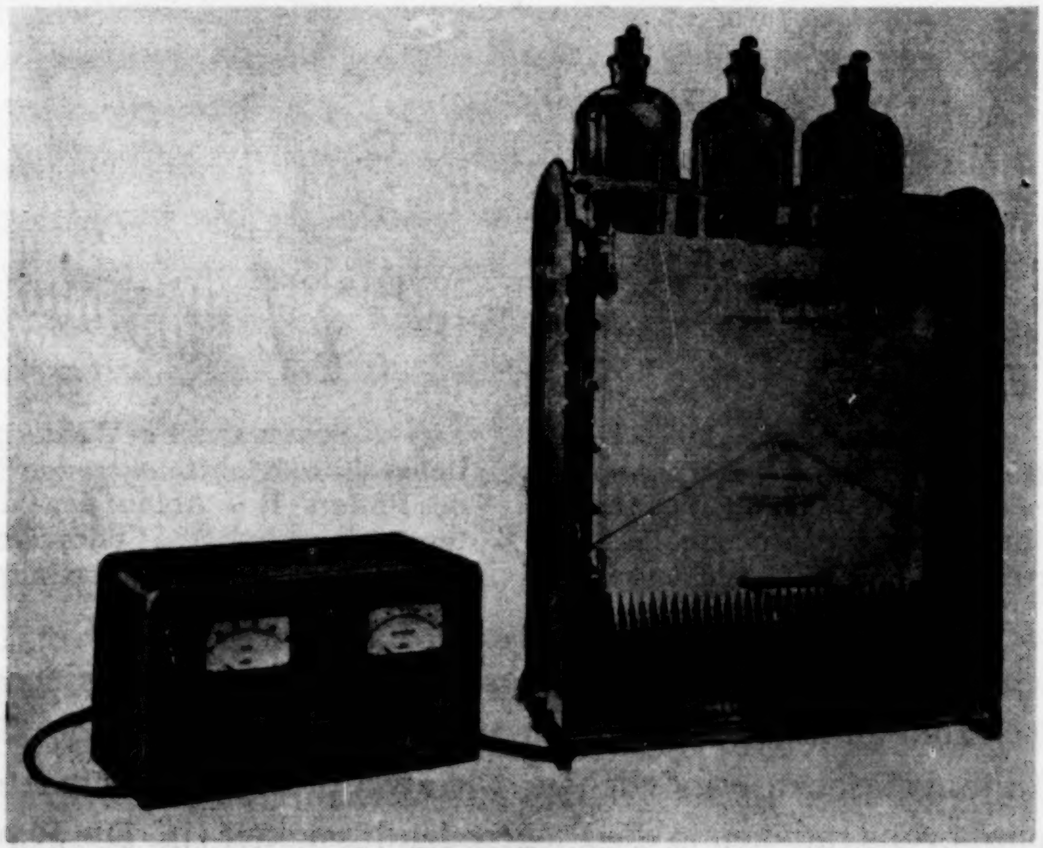


Fig. 3. Gesamtansicht der Apparatur der Firma Shandon, London, bestehend aus der Trennanlage aus Perspex (rechts) und dem stabilisierten Gleichrichter (links).

können. Der Gleichstrom wird einem gesiebten und elektronisch stabilisierten Zweiweggleichrichter mit kontinuierlicher Spannungsregelung von 100 bis 1000 V und maximaler Strombelastung von 25 mA entnommen.

Fig. 4 gibt schematisch die Arbeitsweise der Trennanlage "Spinco Modell CP" der Firma Beckman Instruments, Fullerton, Kalifornien, U.S.A., wieder[27].

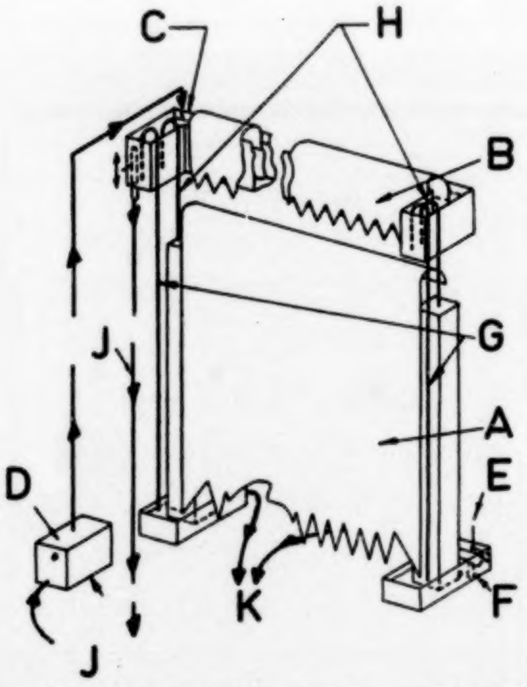


Fig. 4. Schematische Wiedergabe der Trennanlage Spinco Modell CP der Firma Beckman Instruments[27]. A = Trennbogen; B = Vorhang zur Elektrolytversorgung des Trennbogens; C = Elektrolytbehälter; D = elektrisch betriebene Pumpe; E = Platinelektrode; F = Labyrinth-System; G = geeichte Rücklaufkapillaren; H = Ablaufstutzen zur Regulierung des Elektrolytflusses durch die Dochte; I = Elektrolytüberlauf; J = Elektrolyt; K = Mischung, Neuverwendung oder Ablauf.

Durch eine elektrisch betriebene Pumpe wird die Lösung des Grundelektrolyten in den Elektrolytbehälter, bzw. die Pufferwanne befördert. Der eigentliche Trennbogen steht nicht in unmittelbarer Berührung mit den Elektrolyten im Behälter, sondern wird mittels eines besonderen, mit Zipfeln versehenen Vorhangs mit dem Grundelektrolyten versorgt. Dieser zweite Vorhang steht mit dem elektrischen Strom nicht in Berührung. Dieser Umstand verhindert einen übermässigen Verlust des elektrischen Stromes durch die Pufferwanne, was sich in Form einer Shuntwirkung störend auswirken könnte. Eine solche Anordnung ist bereits 1951 von Brattsten und Nilsson[28] vorgeschlagen worden (vergleiche auch Peeters u. Mitarb.[37] und Karler und Deckard[40]). Die Platinelektroden befinden sich in den unten angebrachten Elektrodengefässen, und sind durch ein Labyrinth-System geschützt. Die Produkte der Elektrolyse werden mittels je einer geeichten Rücklaufkapillare aus den Elektrodengefässen mit der Flüssigkeit des Elektrolytbehälters weggespült. Ein weiteres Paar kapillarer Ablaufstutzen versorgt die vertikalen Dochte mit dem Elektrolyten aus dem Elektrolytbehälter. Diese Dochte vermitteln die Stromzufuhr zum Trennbogen, und da sie einen relativ kleinen elektrischen Widerstand aufweisen, wird das Abfallen der elektrischen Feldstärke am Trennbogen in der Richtung von unten nach oben wesentlich verhindert. Ein Elektrolytüberlauf sorgt für ein konstantes Niveau im Elektrolytbehälter.

Eine Gesamtansicht der Apparatur ist in Fig. 5 wiedergegeben. Die effektive Breite des Trennbogens ist 33 cm, die effektive Höhe 34 cm. Die Zahl der Fraktionen ist 16 oder wahlweise 32. Die Umlaufpumpe ist in das Stativ eingebaut. Der Spinco Constat Gleichrichter (nicht abgebildet) erlaubt einen Betrieb mit konstanter Spannung von 100 bis 1000 V, oder auch, wahlweise, ein Arbeiten mit konstanter Stromstärke im Intervall zwischen 10 und 100 mA. Auf Wunsch wird auch ein spezialgebauter Kühlschrank mit doppelwandiger Glastüre geliefert, in dem das Modell CP untergebracht werden kann. Dieses ermöglicht das Arbeiten bei niedrigen Temperaturen bis zu 4°. Ausserdem ist als Zusatz auch ein automatischer Fraktionssammler vorgesehen, der bis zu 6 Sätze von Reagenzgläsern aufnehmen kann. Der Wechsel der Sätze kann in Abständen von 1, 2, 3, 4, 6 oder 12 Stunden erfolgen. Damit ergibt sich eine wartungsfreie Gesamtversuchsdauer von 72 Stunden.

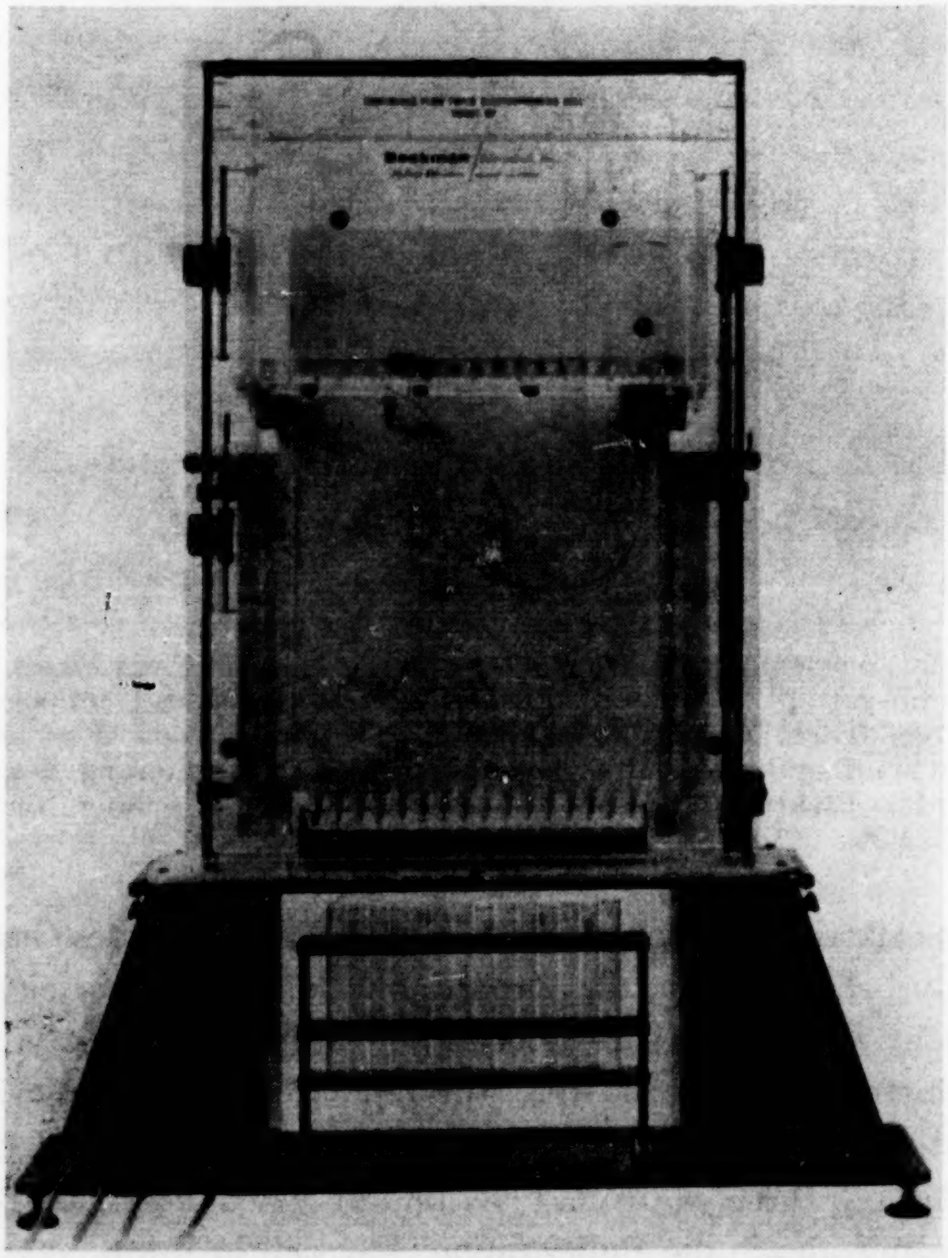


Fig. 5. Gesamtansicht der Trennanlage Spinco Modell CP der Firma Beckman Instruments[27]. Das Energieversorgungsgerät Constat ist nicht abgebildet.

Ein Gerät nach dem klassischen Vorbild der Durrumschen Anordnung (vergl. Fig. 1) wird auch von der Firma Jouan, Paris, hergestellt[29]. Es werden zwei Geräte aus Plexiglas geliefert, das kleinere mit 35 und das grössere mit 52 Auffanggläschen. Die stabilisierte Spannung wird stufenweise zu je 150 V von 150 V bis 900 V geregelt. Ein ähnliches Gerät wurde auch von OSTROWSKI UND NIEWIAROWSKA-PAWLUS beschrieben[30]. Die Abmessungen des Trennbogens sind: Breite 36 cm und Höhe 32 cm. Die beiden Elektrodengefässe sind in je zwei durch einen Elektrolytschlüssel verbundene Gefässe unterteilt. In die vorderen Gefässe tauchen die unteren Enden des Trennbogens, während sich in den dahinterliegenden die Platinelektroden befinden, die intensiv mit frischer Elektrolytlösung bespült werden.

Eine interessante Mikroapparatur wurde unter dem Namen "Mikroelektrochromatophoretisches Gerät" (micro electrochromatophoresis unit) von KARLER, BROWN UND KIRK[31] beschrieben.

Das Gerät (Fig. 6) soll universal verwendbar sein, d.h. für Chromatographie, analytische Papierstreifenelektrophorese, für die Kombination der beiden Techniken, für die zweidimensionale Elektrochromatographie, und schliesslich für die präparative kontinuierliche Elektrophorese im Mikromasstab. Die komplette Apparatur mit dem eingebauten Gleichrichter ist 19.5 cm hoch, 17.5 cm breit und 13.5 cm tief. Der Trennbogen wird auf eine Kunststoffplatte (Grösse 7.5 × 10 cm) aufgelegt. Die Kunststoffplatte ist mit zahlreichen pyramidalen Erhebungen ausgestattet, wegen Verminderung der Kontaktfläche mit dem feuchten Papier. Die Kunststoffplatte samt Trennbogen kann horizontal oder auch um einen Winkel von 10° oder 23° fixiert werden. Der Trennbogen wird, wie aus Fig. 6 ersichtlich, zugeschnitten. Das

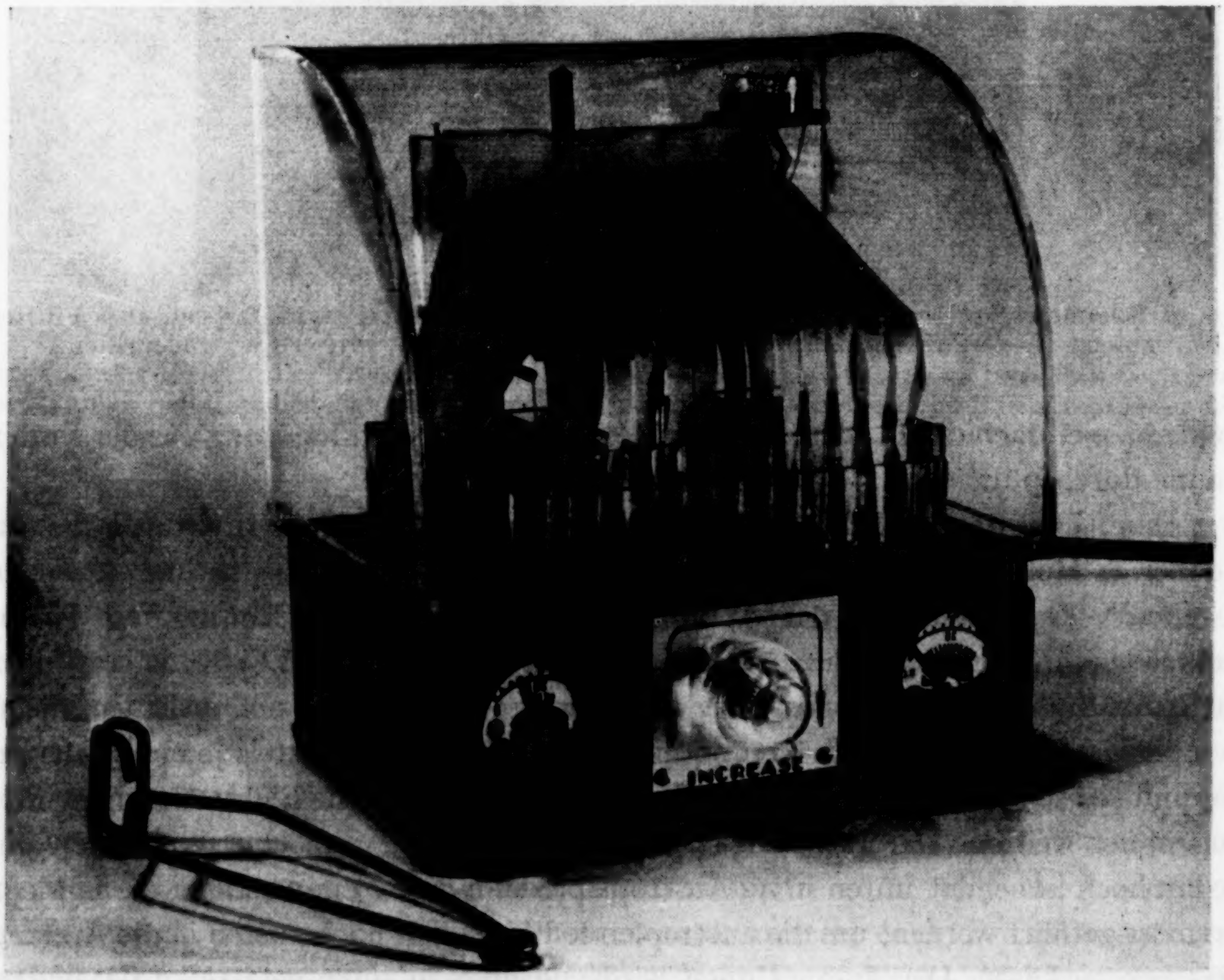


Fig. 6. Gesamtansicht des Mikroelektrochromatographischen Geräts mit eingebauter Stromquelle nach KARLER u. Mitarb.[31]. (Hersteller: Karler Laboratories, Inc., Berkeley, Calif., U.S.A.)

rechte und linke Ende wird nach unten umgebogen, und taucht in die Elektrodengefässe. Die drei oberen Zungen tauchen in die Pufferwanne, und die unteren Zipfel gestatten bei kontinuierlicher Arbeitsweise 13 Fraktionen in den Auffanggläschen zu sammeln. Die Trennanlage wird, um eine feuchte Kammer zu bilden, mit einer Glocke aus glasklarer plastischer Masse zugedeckt. Eine etwas grössere Apparatur ähnlicher konstruktion mit 22 Auffanggläsern wurde auch von Karler angegeben[32,21].

(*ii*) *Apparate, die nach dem Vorbild der Anordnung von Grassmann und Hannig entworfen wurden.* Die Anordnung von Grassmann und Hannig[20,33] unterscheidet sich von der Durrumschen Anordnung im allgemeinen dadurch dass die Elektroden in Form eines Platindrahtes an den beiden Seiten des Trennbogens vertikal angebracht sind. Zur raschen Entführung der Elektrolysenprodukte werden die in besonderen Rillen liegenden Platindrahtelektroden in der Richtung von unten nach oben mit Puffer, bzw. Grundelektrolytlösung aus den beiden seitlichen Mariotteschen Flaschen durchspült.

Eine schematische Darstellung der Anordnung von Grassmann und Hannig gibt Fig. 7 wieder. In den älteren Veröffentlichungen[5,34] wurden die Platindrahtelektroden einfach durch aufgelegte Wattewülste mit der Pufferlösung aus der Pufferwanne durchspült.

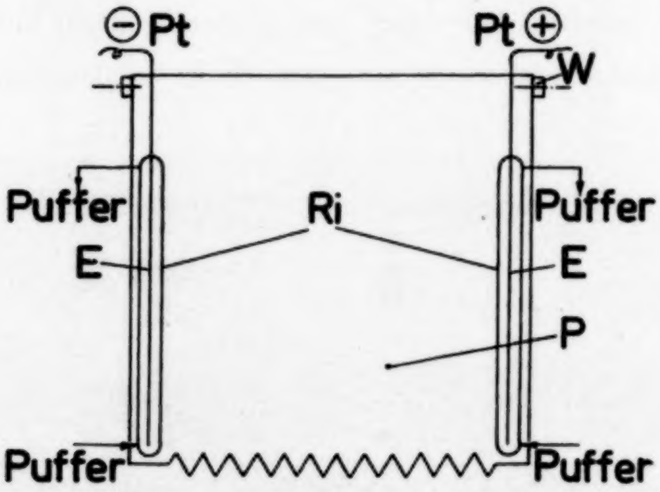


Fig. 7. Schematische Wiedergabe der Anordnung von Grassmann und Hannig. P = Filtrierpapier; W = Pufferwanne; Pt = Platindrahtelektroden; Ri = Elektrodenrillen.

Eine Gesamtansicht der Trennapparatur "Elphor Va", hergestellt von der Fa. Bender & Hobein, München, nach Grassmann und Hannig ist in Fig. 8 wiedergegeben[35]. Der Hauptteil der Apparatur besteht aus einem Einsatz mit Platinelektroden. Dieser Trennrahmen aus einem Polyvinylchlorid (PVC)-Kunststoff dient zur Aufnahme des Trennbogens. Die Platinelektroden befinden sich in Rillen, die an den beiden Seitenflächen des Trennrahmens eingefräst sind. Mittels je eines schaumgummibelegten Elektrodenbalkens kann der Trennbogen an die Elektrodenrillen dicht angepresst werden. Der Trennbogen, dessen wirksame Trennfläche 42 cm breit und 40 cm hoch ist, endet unten in 42 Austropfspitzen mit je 1 cm Abstand, die in kleine Trichter geführt werden, um die austropfende Flüssigkeit nach aussen in die Auffanggläschen zu leiten. Der Trennrahmen wird von oben in einen gekitteten Glaskasten eingelegt, der dann zusammen mit dem am oberen Teil des Trennrahmens befindlichen Glasdeckel eine feuchte Kammer bildet. Auf dem rückwärtigen Teil des Grundgestells sind die drei Vorratsbehälter für die Pufferlösung angebracht. Nach dem Mariotteschen Prinzip bewirken diese die gleichmässige Durchströmung des Elektrodensystems und des Trennbogens mit der Pufferlösung. Unterhalb der Glasküvette ist auf einer heb- und senkbaren Bühne das Vorlagegestell mit den darin befindlichen Auffanggläsern untergebracht. Diese Auffanggläser dienen zur Aufnahme der getrennten Komponenten des Stoffgemisches.

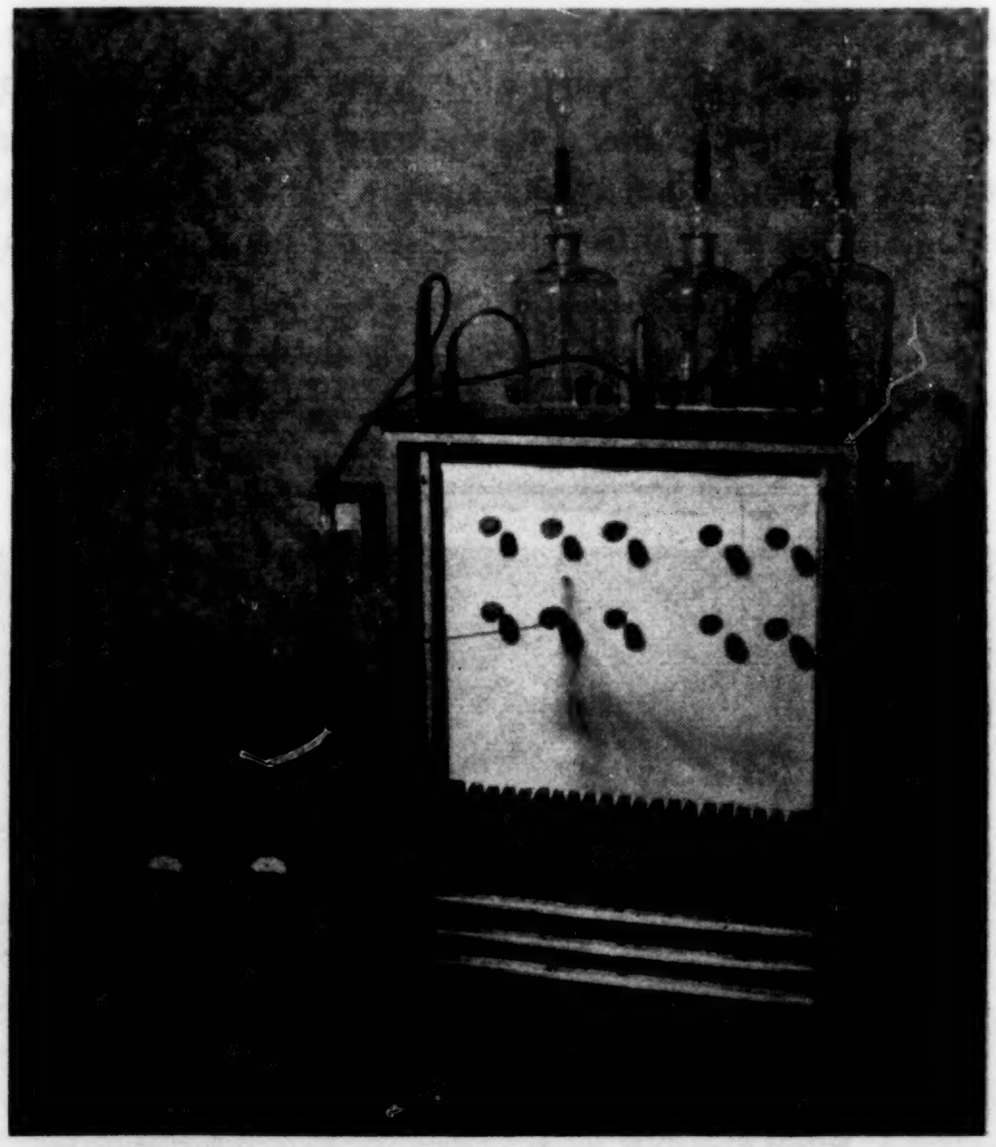


Fig. 8. Gesamtansicht der Trennapparatur "Elphor Va"[35] nach GRASSMANN UND HANNIG. Links am Rahmen befestigt befindet sich die elektrisch betriebene automatische Dosierpumpe für das zu trennende Substanzgemisch. (Hersteller: Fa. Bender & Hobein, München, Deutschland.)

Als Stromquelle dient ein Gleichrichter, der die stufenlose Regulierung der Stromspannung bis zu 800 V und eine maximale Stromstärke von 40 mA erlaubt. Das zu trennende Substanzgemisch kann wahlweise an mehreren Stellen durch Öffnungen in der Frontglaswand mittels einer elektrisch betriebener automatischen Dosierpumpe dem Trennbogen zugeführt werden (vergl. Fig. 8). Diese Dosierpumpe[35] arbeitet nach dem Prinzip eines leerlauffreien Spritzensystems, bei dem der kontinuierliche Vorschub des Kolbens einer Injektionsspritze (1, 2, 3, 4 und 5 mm pro Stunde) durch einen elektrischen Synchronmotor mittels einer Präzisionsgetriebe erreicht wird. Die Dosiergeschwindigkeit kann durch Veränderung des Querschnittes der Injektionsspritze und der Vorschubgeschwindigkeit des Glaskolbens in weiten

Grenzen variiert und während des Versuches vollkommen konstant gehalten werden. Das Volumen der dosierten Substanz je Zeiteinheit ist jederzeit durch die verwendete Injektionsspritze und den gewählten Kolbenvorschub bekannt.

Eine Modifikation der Grassmannschen Apparatur wurde von PUČAR[36] angegeben (Fig. 9 und 10).

Es wurden einige besonderen Vorteile dieser Apparatur hervorgehoben.

Das Filtrierpapier kann von aussen jederzeit und ohne Unterbrechung der Elektrophorese nachgespannt und zentriert werden. Infolgedessen kann das Papier trocken montiert werden, und, nach selbsttätiger und gleichmässiger kapillarer Anfeuchtung aus der Pufferwanne, nachgespannt werden. Das Filtrierpapier kann beim Montieren an die Elektrodenrinnen mittels zwei mit Schaumgummi belegten

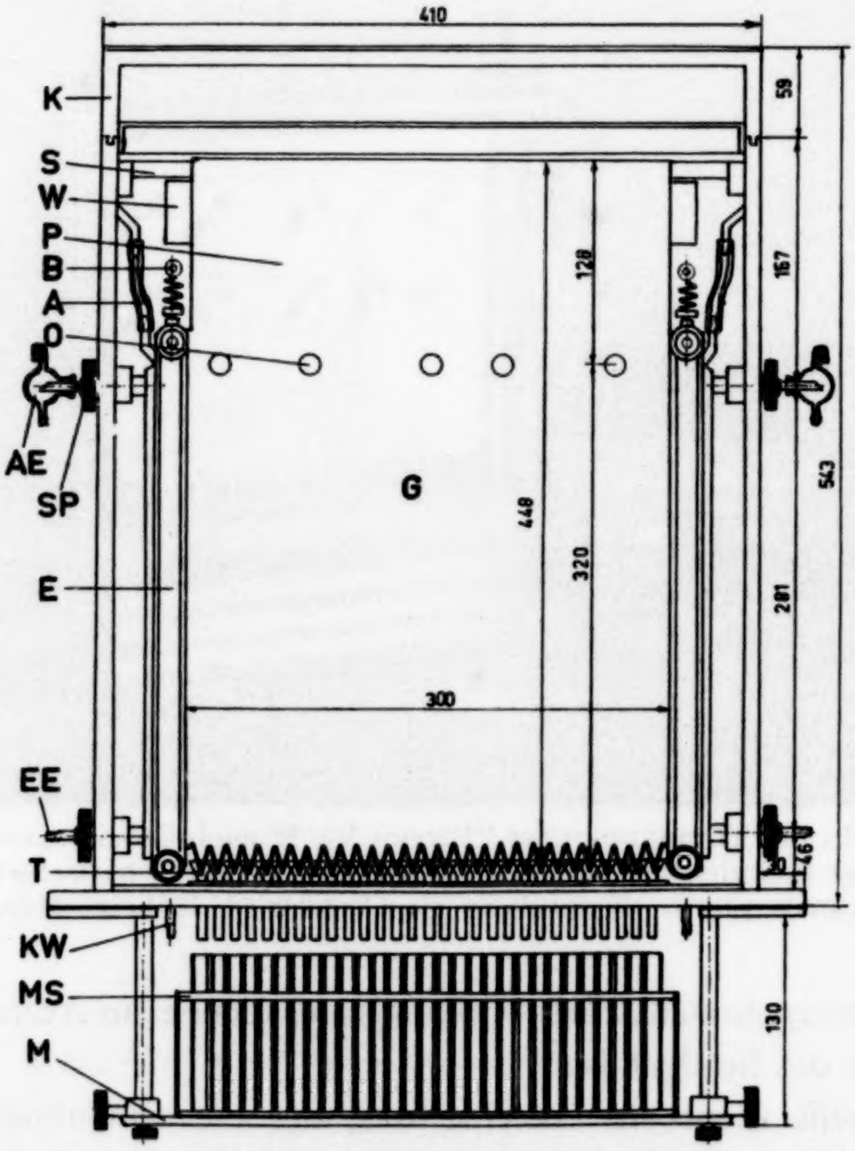


Fig. 9. Frontansicht der feuchten Kammer nach PUČAR[36]. Baumaterial geschweisstes PVC. G = vordere Glaswand; K = abnehmbare Kappe aus PVC; S = abnehmbarer Glasstab bzw. abnehmbares Glasrohr zur Unterstützung des Filtrierpapiers; W = Pufferwanne aus PVC; P = Filtrierpapier; B = Stromzufuhrbüchse mit Stromzuleitung; A = Gasableitung; O = Öffnungen in der Glaswand für die Zufuhrvorrichtung für das zu trennende Substanzgemisch; E = schaumgummibelegter Elektrodenbalken aus Metall mit zwei Rändelmuttern zum Anpressen des Filtrierpapiers. Unter dem Elektrodenbalken befindet sich die Elektrodenrinne aus PVC mit der Platindrahtelektrode. SP = Spannvorrichtung und Zentriervorrichtung aus PVC für das Filtrierpapier; AE = Ausgang der Elektrodenspülung mit dem Tropfenzähler aus Glas; EE = Eingang der Elektrodenspülung. Der Eingangstropfenzähler aus Glas befindet sich an jeder der drei Mariotteschen Flaschen von je 5 l Inhalt. T = Auffangtrichter für die getrennten Komponenten; KW = Abfuhr des Kondenswassers; M = Metalltasse mit Vorrichtung zur Einstellung der Gläschenhöhe; MS = abnehmbares Metallstativ mit 29 Auffanggläschen von je 21 ml Inhalt.

Elektrodenbalken und vier Rändelmuttern gleichmässig angepresst und gedichtet werden. Beim Montieren und Demontieren wird die vordere Glaswand nach der Entfernung der oberen Kappe einfach nach oben ausgezogen, so dass die ganze Papierebene, bzw. das Papier, vollkommen frei für Manipulationen bleibt. Wenn die Glaswand dann, beim Einschieben, um die Vertikalachse um 180° gedreht wird, fallen die fünf Öffnungen für die Substanzzufuhrvorrichtung auf weitere vier Stellen, so dass mit den neuen Zuführungslagen, ohne Wechsel der Stromrichtung, jeweils die günstigste Zuführungsstelle für die zu trennende Substanz bereit steht. Die Elektrodenrinnen haben eine besondere Gasableitung nach aussen, so dass das Abtropfen der Pufferlösung am Ausgang der Apparatur nicht verhindert wird, und ausserdem können schädliche Gase, die sich an den Elektroden entwickeln, wie z.B. Chlor, nicht mit dem Papier und der zu trennenden Substanz in Berührung kommen. Da beim Papierwechsel keine Gefahr der Beschädigung des Papiers besteht, kann die Apparatur entsprechend grösser gebaut werden, und ausserdem können auch dünnere und weniger feste Papiersorten Verwendung finden.

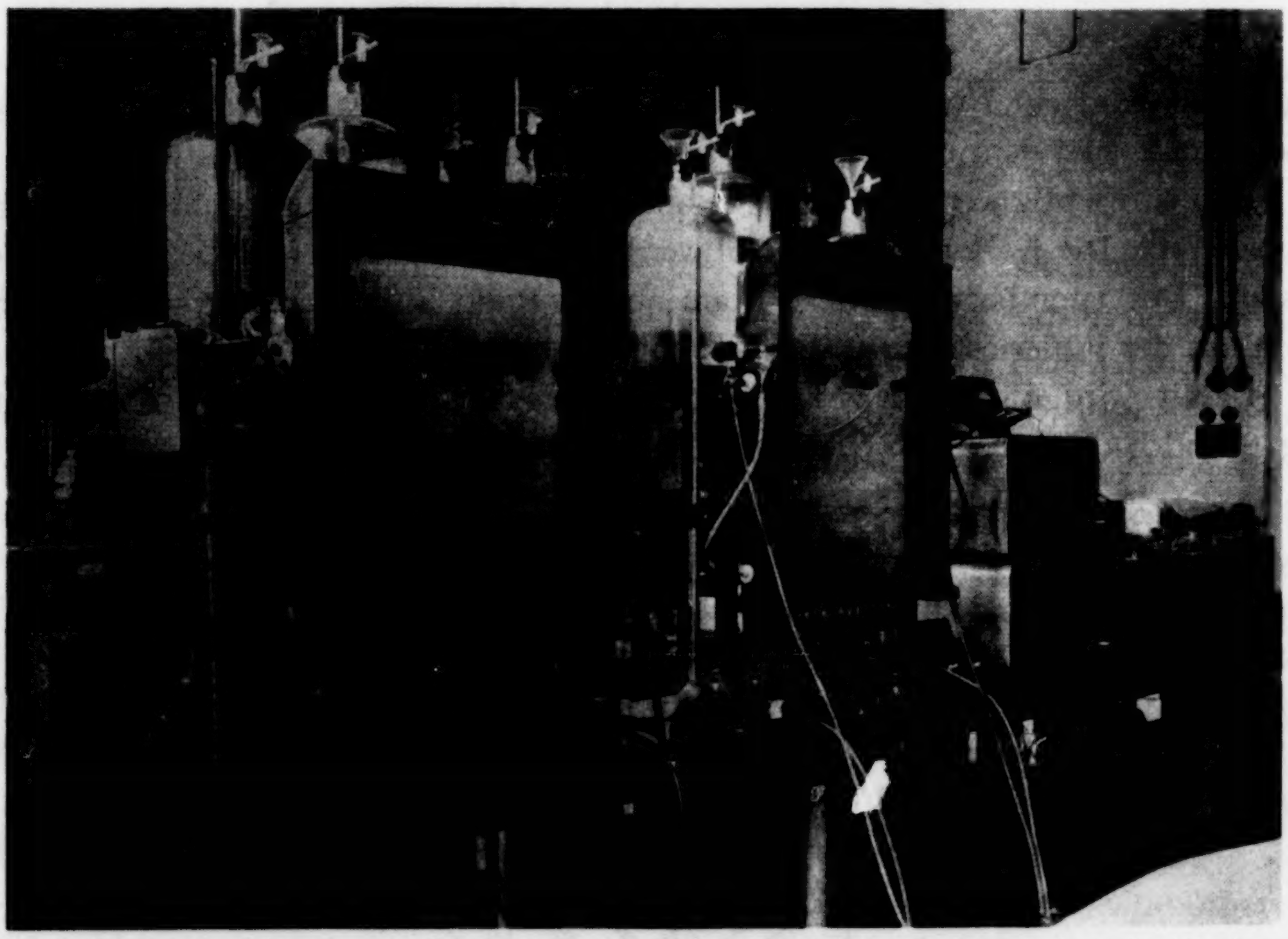


Fig. 10. Gesamtansicht an zwei Trennapparaturen im Betrieb im Laboratorium des Verfassers.

Als Stromquelle wird ein Gleichrichter mit stufenloser Regulierung der Stromspannung von 100 bis 1000 V, und einer maximalen Strombelastung von 50 mA verwendet.

Neulich haben PEETERS, VUYLSTEKE UND NOË[37] eine Apparatur mit Kaskadenelektroden beschrieben (Fig. 11). Die Kaskadenelektroden erhalten einen identischen hydrostatischen Druck durch die ganze Höhe der vertikalen Elektroden aufrecht,

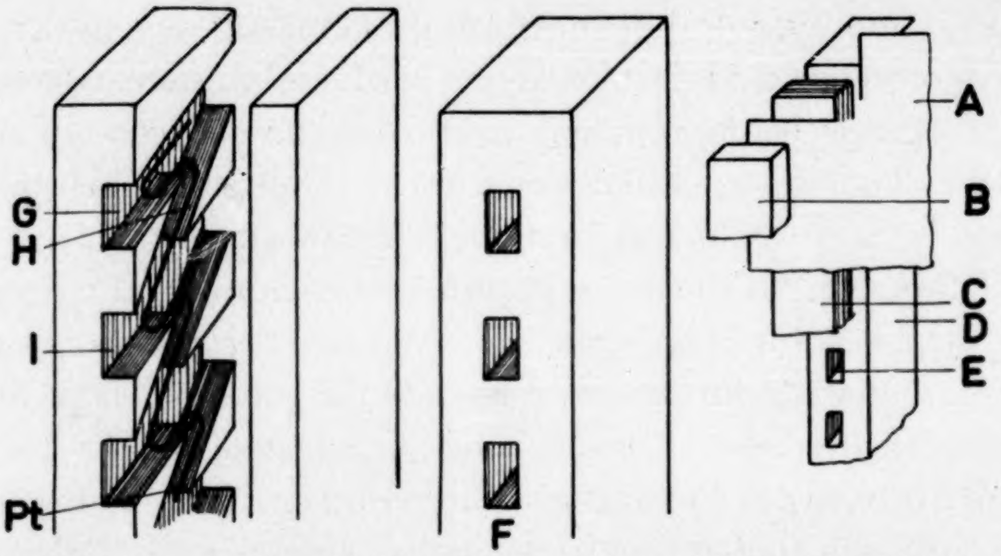


Fig. 11. Kaskadenelektroden (schematisch) nach PEETERS u. Mitarb.[37]. Rechts ist die Befestigung des Trennbogens ersichtlich. Der Trennbogen A ist zwischen den Balken B und den Elektrodenträger D gepresst. Der Kontakt mit dem Puffer kommt durch das Fenster E zustande. Einige Papierstreifen C vermitteln den Kontakt zwischen dem Elektrodenträger und dem Trennbogen. In der Mitte der Abbildung ist der Elektrodenträger F wiedergegeben. Links ist der vertikale Schnitt durch den Elektrodenträger ersichtlich. Die Pufferlösung wird in die erste Kammer G geleitet, kommt durch das Fenster in Berührung mit dem Trennbogen, und fliesst nachdem die Kammer aufgefüllt ist, durch den Überlauf H in die nächste Kammer I, und so weiter. Pt = Platindrahtelektrode.

und somit wird ein Druckgleichgewicht zwischen dem Elektrolyten, der die Elektroden spült, und dem Elektrolyt am Trennbogen aufrechterhalten.

Dies ist wichtig wenn man ohne Celophanmembranen arbeitet (vergl. den theoretischen Teil). Die Kaskadenelektroden werden in der Richtung von oben nach unten bespült. Der Trennbogen wird auch am oberen Rand in Zacken zugeschnitten, die dann mit der Pufferlösung aus der Pufferwanne betropft werden (Fig. 12).

Damit wird die Shuntwirkung der Pufferwanne vermieden (vergleiche auch Fig. 4 und BRATTSTEN UND NILSSON[28]).

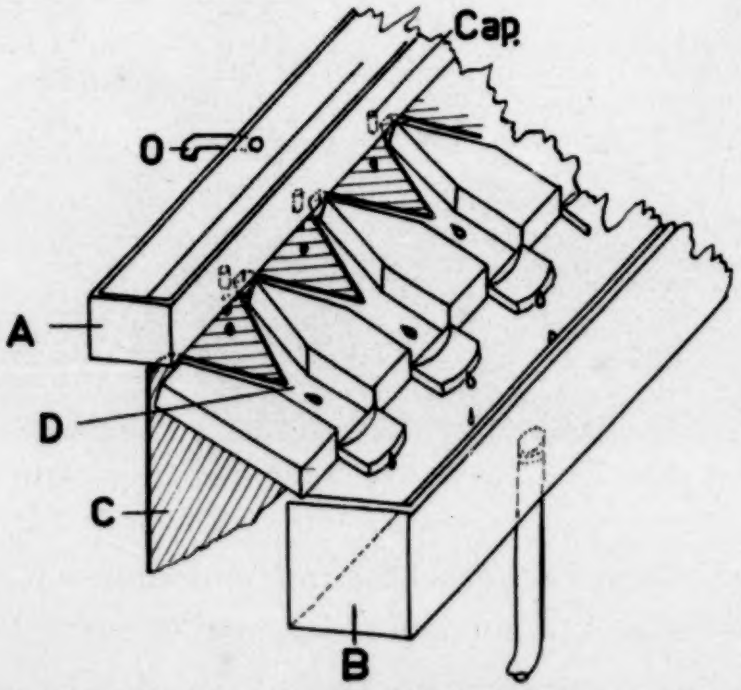


Fig. 12. Das Berieselungssystem des Trennbogens nach PEETERS u. Mitarb.[37]. Aus der mit einem Überlauf O versehenen Pufferwanne A tropft die Pufferlösung über Kapillaren (Cap.) auf die aus dem oberen Rand des Trennbogens C ausgeschnittenen Zacken. Jeder Zacken des Trennbogens liegt in einem separaten Kanal D. Die überflüssige Flüssigkeit wird durch eine Sammelrinne B aus allen Kanälen entführt.

Mit den Apparaturen die ohne Kühlung in feuchter Kammer arbeiten, kann man praktisch maximal eine Stromleistung von 40–50 W erreichen. Wenn man, um die Durchsatzleistung zu erhöhen, von Filtrierpapieren zu dicken Filtrierkartons übergeht, und dabei mit Substanzen arbeitet, die kleine elektrophoretische Beweglichkeiten aufweisen, steigt die erforderliche Stromleistung stark an (auf 250–500 W). Die Joulesche Wärmeentwicklung wird dann die Temperatur des Trennbogens und die Verdunstung erheblich erhöhen. Diese Verdunstung wird sich dann sehr störend auf den Trennprozess auswirken (siehe Abschnitt 3 (a)), und es könnte ausserdem gelegentlich zum Abtrocknen oder sogar auch Durchbrennen des Trennbogens kommen. Ein Zusatz von 15–20% Glycerin oder Propylenglykol zur Pufferlösung verhindert nach einem Vorschlag von DURRUM[6] die Verdunstung und das Abtrocknen des Trennbogens, aber um so mehr steigt dann die Temperatur des Trennbogens an. Ausserdem ist es bei präparativen Arbeiten nicht immer einfach, das Glycerin aus den gesammelten Fraktionen zu entfernen. Im allgemeinen hat es sich gezeigt, dass das Einlegen des Trennkartons zwischen gekühlte Glasplatten nicht die gewünschte Trennschärfe der Bahnen ergibt, und zwar wegen anderer Wanderungsverhältnisse an der Berührungsstelle Flüssigkeit–Glas als im Inneren des Trennbogens.

Neulich hat HANNIG[33,38] eine besondere Apparatur entwickelt, die auf Filtrierkartons in feuchter Kammer mit grossen Stromleistungen arbeitet (Fig. 13 und 14).

Der Trennkarton wird durch einen Kaltluftstrom von — 10 bis — 20° von der einen Seite gekühlt, während gleichzeitig Flüssigkeit, im Masse der Verdampfung, von der anderen Seite auf die gekühlte Trennfläche aufkondensiert wird, und zwar über einen zweiten elektrisch beheizten feuchten Karton. Dieser Karton befindet sich vor einer Glasplatte, die durch isolierte Heizdrähte über einen Regeltransformator auf die gewünschte Temperatur gebracht werden kann. Für das Aufkondensieren genügt eine Temperaturdifferenz von 5° zwischen Trennkarton und beheizbarem Karton. Die Kühltemperatur und der Grad der Aufdampfung kann durch die Leitfähigkeit des Trennsystems automatisch gesteuert werden. Bei den verhältnismässig grossen Stromstärken müssen die Elektroden rasch durchspült werden (*ca.* 0.5 l/Min.). Dies erfolgt durch eine Umlaufpumpe. Die aus den Elektrodenräumen austretenden Pufferlösungen werden in das Vorratsgefäss zurückgeleitet und dort wieder miteinander vermischt.

Die Gesamtansicht einer vervollkommneten Apparatur mit Kaltluftkühlung und Aufkondensieren nach HANNIG[39] gibt Fig. 14 wieder. Die Abmessungen der Trennkammer entsprechen denen der "Elphor Va". Es wird ein stromstabilisierter Gleichrichter verwendet, der eine maximale Spannung von 1500 V und eine maximale Stromstärke von 300 mA gestattet. Die Trennleistung beträgt *ca.* 8–10 g Protein pro Tag.

KARLER UND DECKARD[40] haben eine Apparatur mit vertikalen Durchflusselektroden angegeben, die im Inneren der feuchten Kammer mit einem Kühltank versehen ist. Dieser Kühltank wird mit einer Mischung von festem CO_2 und einem organischen Lösungsmittel beschickt. Die Luftzirkulation im Inneren der feuchten Kammer wird durch einen Ventilator aufrechterhalten.

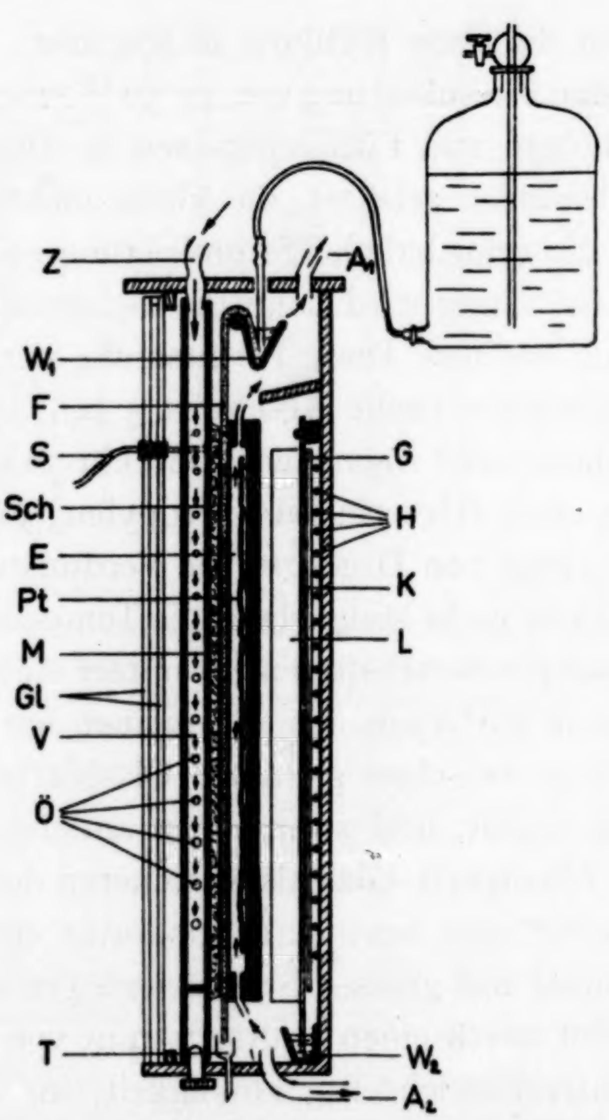


Fig. 13. Eine Weiterentwicklung der "Elphor Va" Apparatur für Trennungen am Filtrierkarton unter Kühlung mit einem Kaltluftstrom von — 10° bis — 20° und gleichzeitigem Aufkondensieren von Wasserdampf nach HANNIG[38]. Trennkammer im Seitenschnitt schematisch. W_1 = Pufferwanne; F = Filtrierkarton (Trennbogen); Sch = Schaumgummiauflage; E = Elektrodenräume; Pt = Platinelektroden; M = Membrane aus Cellophan; Gl = Abnehmbare Glasscheiben (Vorderwand); S = Zuführung der Trennsubstanz; T = Trichter; Z = Zuführung des Kaltluftstromes; V = Verteilungsrohr für Kaltluft, gleichzeitig Andruckleiste; Ö = Luftaustrittöffnungen in Richtung Trennkarton; A_1, A_2 = Abluft; G = beheizbare Glasplatte; H = isolierte Heizdrähte; K = Aufdampfkarton mit Lamellen L; W_2 = Flüssigkeitswanne, gefüllt mit Wasser.

Eine Apparatur mit grossem Durchsatz und eingebauter Kühlmaschine neuester Bauart nach KARLER[41] gibt Fig. 15 in Gesamtansicht wieder.

(*iii*) *Durch Zentrifugalkraft beschleunigte kontinuierliche Elektrophorese in feuchter Kammer nach McDonald.* Statt der Schwerkraft in der Richtung der Bewegung des Elektrolyten im Trennbogen benutzen McDONALD und Mitarbeiter[42–45] die Zentrifugalkraft, zu der, wie üblich, senkrecht ein elektrischer Strom fliesst. Die horizontal rotierende feuchte Kammer (Fig. 16) ist aus zwei flachen rechteckigen Tassen zusammengesetzt, zwischen denen in der horizontalen Ebene ein Trennbogen der Grösse 56 × 23 cm aufgespannt ist. Die untere Tasse ist aus Bakelit und die obere aus Lucit hergestellt.

Die Platindrahtelektroden befinden sich längs der zwei längeren Enden des Trennbogens. Die kürzeren Enden des Trennbogens sind zickzackförmig zugeschnitten. Jeder Zacken führt zu einem Auffangglas, deren sich neun auf jeder Seite befinden. Durch zwei Messingschleifringe, die an der unteren Seite der unteren Tasse angebracht sind, wird über Schleifbürsten der Gleichstrom (Spannung zwischen 200 und 1000 V) den Platinelektroden zugeführt. Das ganze System rotiert in der horizontalen Ebene, durch einen Elektromotor angetrieben, mit einer Geschwindigkeit von 400 bis 900 Umdrehungen pro Minute. Die kontinuierliche Zufuhr des Grundelektrolyten und der zu trennenden Substanz wird in der Nähe der Rotationsaxe durch eine besondere elliptische Zufuhrvorrichtung aus nichtrostendem Stahl ermöglicht (Fig. 17).

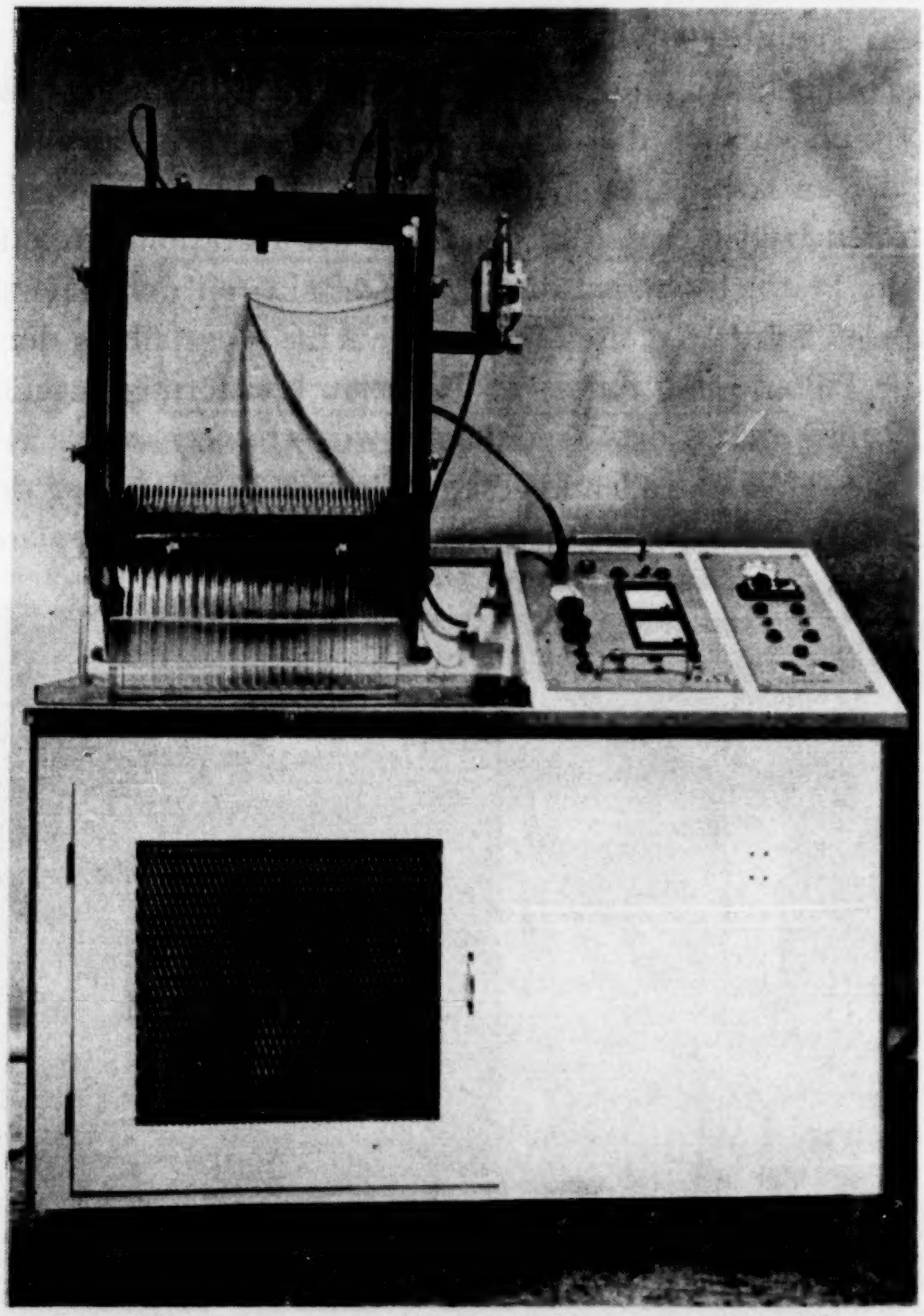


Fig. 14. Gesamtansicht der Trennanlage "Elphor VaP" nach HANNIG[39] mit Kaltluftkühlung. Trennleistung 8–10 g Protein pro Tag. Stromstabilisierter Gleichrichter bis 1500 V und 300 mA (Hersteller: Fa. Bender & Hobein, München, Deutschland.)

Diese Zufuhrvorrichtung besteht im wesentlichen aus einer oval geformten flachen Rinne (grössere Axe 9.5 cm, kleinere Axe 9 cm), deren äussere Wände nach innen geneigt sind. Die grössere Axe der Vorrichtung läuft parallel zur längeren Axe der feuchten Kammer, und an jedem ihrer Enden befindet sich eine kapillare Öffnung, durch die die zu trennende Substanz dem Trennbogen kontinuierlich zugeführt wird. Die Zufuhrvorrichtung ist an die Rotationsaxe des Systems durch zwei Speichen fixiert, und rotiert samt der feuchten Kammer und dem Trennbogen.

Die zu trennende Substanz wird durch eine stillstehende Glaskapillare kontinuierlich dem hohlen rinnenförmigen elliptischen Ring zugeführt, und fliesst durch die zwei kapillaren Öffnungen der Zufuhrvorrichtung dem Trennbogen kontinuierlich zu. Die Pufferlösung wird durch eine zweite ebenfalls stillstehende Glaskapillare etwas näher dem Rotationszentrum, innerhalb des elliptischen Ringes der Zufuhrvorrichtung, direkt dem Trennbogen zugeführt. Es entstehen immer zwei untereinander identische spiegelbildliche Trennungen. Bei den diskontinuierlichen elektrochromatographischen Trennungen fällt die elliptische Zufuhrvorrichtung und die Kapillare für die Zufuhr der zu trennenden Substanz weg. Die Substanz wird dann einfach punktförmig an den beiden Seiten in der Nähe der Rotationsaxe aufgetragen. Die Tatsache, dass die Platinelektroden direkt in Kontakt mit dem Trennbogen stehen, macht keine grossen Schwierigkeiten in Bezug auf die Wegspülung der Elektrolysenprodukte. Da die Pufferlösung durch die Rotation kreisförmig zugeführt wird, und sich auch kreisförmig durch den Trennbogen weiterbewegt, wird sie in grösserem Masse in der Nähe der Elektroden angesammelt und fliesst entlang der Elektroden, die Elektrolysenprodukte wegspülend, in die vier äusseren Auffanggläschen. Um den

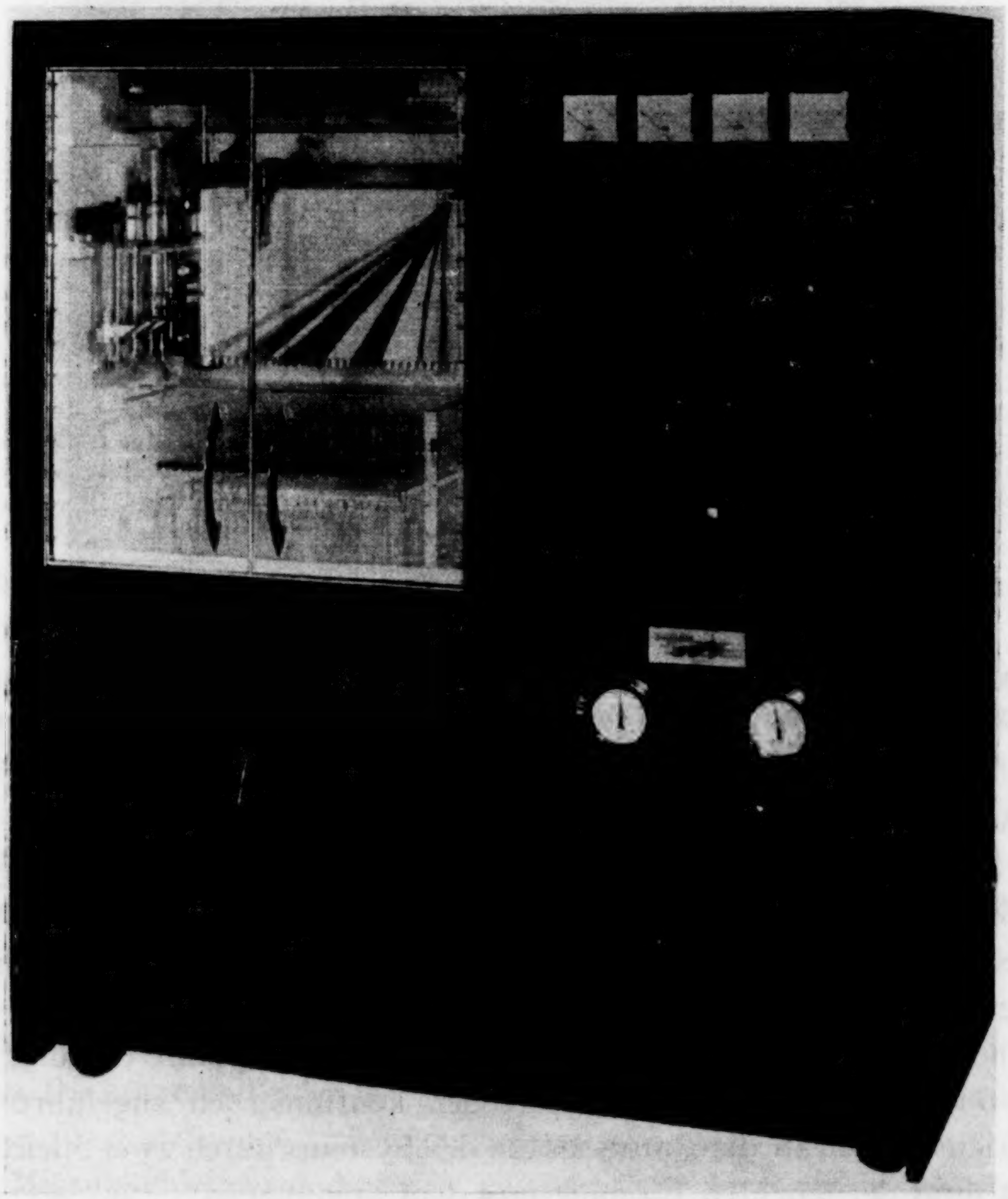


Fig. 15. Gesamtansicht einer neueren Trennapparatur mit grosser Leistung nach KARLER[41]. (Hersteller: Karler Laboratories, Inc., Berkeley, Calif., U.S.A.)

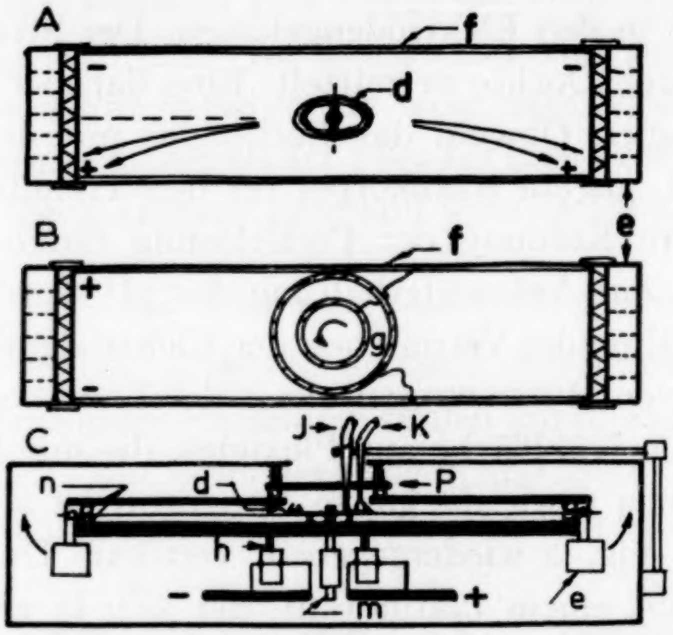


Fig. 16. Apparatur für durch Zentrifugalkraft beschleunigte kontinuierliche Elektrophorese nach McDonald u. Mitarb.[45]. A = obere Seite des Rotors; B = untere Seite des Rotors; C = Seitenansicht der Apparatur; d = ovale Zufuhrvorrichtung; e = Auffanggläschen, die beim Rotieren aufgeschwungen werden; f = Platindrahtelektroden; g = Messingschleifringe; h = Graphitschleifbürsten; j = Kapillare für die Pufferzufuhr zum Trennbogen; k = Kapillare für die Zufuhr der zu trennenden Substanz; m = Antriebsachse des Rotors; n = Dichtungen aus Silikon; p = nichtrotierende Dichtungsanlage.

Dampfgleichgewichtzustand der feuchten Kammer aufrechtzuerhalten, wird auf den Boden der feuchten Kammer ein sehr dickes, mit Pufferlösung befeuchtetes Blatt Filtrierpapier montiert.

(*iv*) *Anordnungen in feuchter Kammer mit einem Trennbogen, der einerseits mit einer Glas- oder Kunststoffwand in Berührung steht.* Eine sehr einfache Apparatur, die man im Laboratorium selbst zusammenstellen kann, hat Saroff[46] beschrieben. Der Trennbogen wird um ein mit Pufferlösung gefülltes Laboratoriumsbecherglas gewickelt. Die Pufferlösung wird aus dem Becherglas über Dochte von oben den Trennbogen zugeführt und unten in den Auffanggläschen gesammelt. Die Platin-

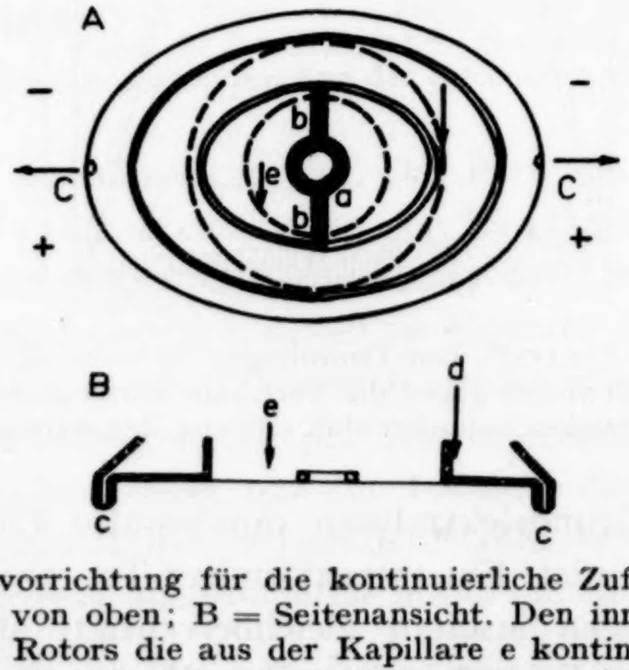


Fig. 17. Elliptische Zufuhrvorrichtung für die kontinuierliche Zufuhr der Trennsubstanz zum Trennbogen[45]. A = Ansicht von oben; B = Seitenansicht. Den inneren gestrichelten Kreis beschreibt beim Rotieren des Rotors die aus der Kapillare e kontinuierlich zuströmende Pufferlösung. Den äusseren gestrichelten Kreis beschreibt beim Rotieren des Rotors die aus der Kapillare d kontinuierlich zuströmende zu trennende Substanz. Durch die zwei kapillaren Öffnungen c wird beim Rotieren des Rotors die zu trennende Substanz kontinuierlich und gleichmässig dem Trennbogen zugeführt.

elektroden befinden sich in den Elektrodengefässen. Der Stromkontakt zum Trennbogen wird ebenfalls durch Dochte vermittelt. Eine darüber aufgesetzte Glasglocke bildet die feuchte Kammer. Obwohl das Becherglas mit der Pufferlösung, infolge grosser Wärmekapazität, als ein Kühlkörper für den Trennbogen dienen kann, hat ADAMIK[47] eine besondere Kühlung der Pufferlösung durch Einlegen einer Kühlschlange vorgeschlagen. Zur Aufrechterhaltung des pH wurde auch ein System beschrieben, das für fortwährendes Vermischen der Elektrodenflüssigkeiten sorgt.

In der Anordnung von HOLDSWORTH[48] liegt der Trennbogen auf einer gegen die Vertikale etwas geneigten Kühlfläche aus Plexiglas, die mit Leitungswasser gekühlt wird. Die feuchte Kammer sowie die ganze Apparatur ist aus Plexiglas gebaut. Es werden, ähnlich wie in Fig. 2 wiedergegeben, vertikale Durchflusselektroden verwendet. Die bestehen aus einem Platindraht, der sich in einem mit Puffer durchspülten Cellophanschlauch befindet. Es wurde auch eine elektrisch betriebene automatische Dosierpumpe für das zu trennende Substanzgemisch beschrieben. Verwendet wird ein Gleichrichter mit stabilisierter Spannung zwischen 70 und 840 V, mit Spannungsregelung in Stufen von je 70 V und maximaler Strombelastung von 30 mA.

Die Apparatur nach STRAIN[49] ist in Fig. 18 wiedergegeben. Der Trennbogen ist trapezförmig nach oben zugeschnitten und liegt in einer feuchten Kammer aus Polyethylen auf einem Block aus Polystyrenschaum, der etwas gegen die Vertikale geneigt ist. Als Elektroden dienen Platindrähte, die entlang der schrägen Seiten des Trennbogens durch die dort befindlichen Löcher hindurchgezogen sind. Gelegentlich werden auch mit dcn Grundelektrolyten durchspülte Flüssigkeitselektroden aus Kollodiumschlauch verwendet. Ein trapezförmiger Trennbogen ist einem parallelogrammförmigen Trennbogen insofern gleichberechtigt, als beim trapezförmigen Trennbogen die elektrische Feldstärke im selben Masse von oben nach unten abfällt wie die Geschwindigkeit des Grundelektrolyten, so dass der Neigungswinkel der Bahnen, im System ohne Verdunstung, konstant bleibt, und geradlinige Bahnen

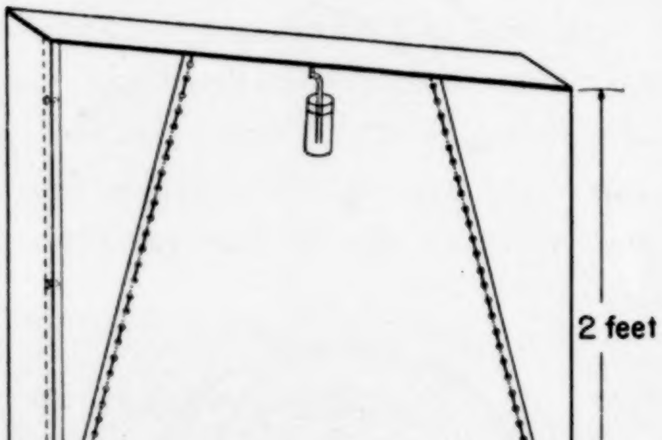


Fig. 18. Die Apparatur nach STRAIN[49]. Der Trennbogen ist trapezförmig zugeschnitten und liegt in einer feuchten Kammer auf einem gegen die Vertikale etwas geneigten Block aus Polystyrenschaum. Die Platindrahtelektroden befinden sich entlang der schrägen Seiten des Trennbogens.

entstehen. Diese Bahnen erscheinen aber wegen der horizontalen Strömungsvektoren im unteren Teil unnötig verbreitet. Im System mit Verdunstung (feuchte Kammer) werden die Verhältnisse etwas verwickelter, aber man wird sich an Hand theoretischen Überlegungen (vergleiche Abschnitt 3 (a)) für jede Arbeitsweise ein entsprechendes Bild über den jeweiligen Trennungsverlauf verschaffen können.

2. *Trennbogen zwischen Glas- oder Kunststoffplatten* (*System ohne Verdunstung*)

STRAIN UND SULLIVAN[7] haben eine sehr einfache Anordnung beschrieben, die man auch jederzeit im Laboratorium Zusammenstellen kann. Fig. 19 zeigt diese einfache "elektrographische Zelle". Ein besonders zugeschnittener und an den schraffierten Stellen paraffinierter Trennbogen wird samt den Platindrahtelektroden zwischen zwei Glasplatten gelegt und zusammengepresst. Das Paraffinieren des Trennbogens wird durch Aufpinseln einer gesättigten Lösung von Paraffin in Kohlentetrachlorid oder in Petrolether und darauffolgende Verdunstung des Lösungsmittels erreicht. Die Platindrahtelektroden befinden sich direkt in Kontakt mit dem Trennbogen, ohne besondere Spülung der Elektroden. Deswegen werden während des Betriebes, besonders in der Nähe der Elektroden, die pH Schwankungen erheblich. Diese elektrographischen Zellen wurden bisher nur für kontinuierliche und diskontinuierliche Trennungen anorganischer Ionen verwendet. Grössere Zellen (32 cm × 32 cm) wurden in einem als Presse ausgebildeten Stahlrahmen montiert und zusammengepresst[50].

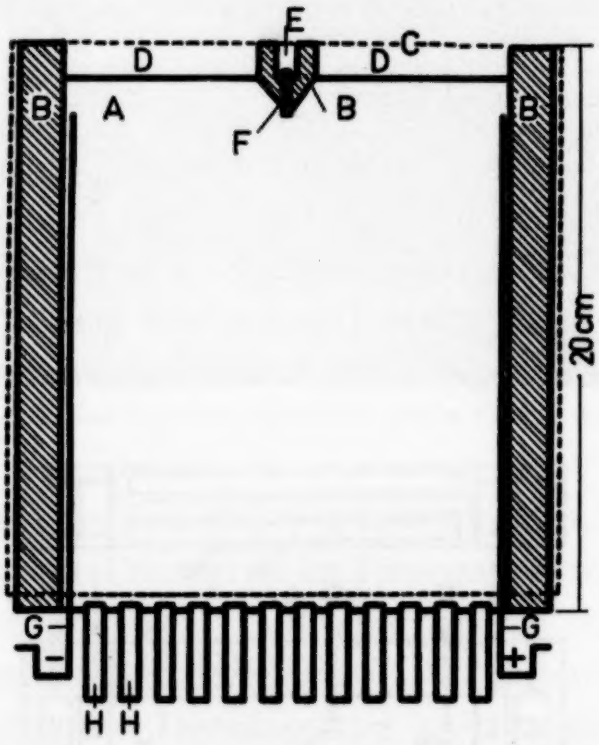


Fig. 19. Die "elektrographische Zelle" nach STRAIN UND SULLIVAN[7]. A = Filtrierpapier; B = die paraffinierten Stellen des Trennbogens; C = Glasplatten; D = ausgeschnittene Teile des Trennbogens, die als Pufferwanne dienen; E = ausgeschnittene Stelle des Trennbogens, die zur Aufnahme der zu trennenden Substanz dient; F = Papierdocht durch die paraffinierte Fläche; G = Platindrahtelektroden in Rillen an der Glasplatte; H = Filtrierpapierstreifen zur Ableitung des Grundelektrolyten und der getrennten Substanz.

Bei der Apparatur nach BRATTSTEN UND NILSSON[28] befindet sich der oben und unten zickzackförmig zugeschnittene Filtrierkarton (Grösse 40 × 20 cm) zwischen zwei 7 mm dicken Glasplatten, die zwecks Unterdrückung der Adhäsion zwischen Flüssigkeit und Glas mit Silikonfett behandelt wurden. An jeder Glasplatte ist an der äusseren Seite zwecks Kühlung eine Küvette aus Plexiglas angebracht, die von Leitungswasser durchströmt wird. Die oberen Zacken des Trennbogens werden durch einen zweiten mit Zacken versehenen Filtrierpapiervorhang mit den Grundelektrolyten aus der Pufferwanne betropft. Durch diese Massnahme wird, wie bereits erwähnt (vergleiche dazu Fig. 4 und 12), die Shuntwirkung der Pufferwanne verhindert. Die vertikalen Platinelektroden werden, je nach Wahl, mit der Grundelektrolytlösung gespült oder es wird auf Spülung verzichtet. Die Stromquelle liefert Gleichspannungen bis 1000 V und Stromstärken bis 250 mA.

(*b*) *Apparate mit küvettenförmiger Trennkammer und antikonvektiver Füllung*

1. *Apparatur nach Grassmann und Hannig*

Eine Weiterentwicklung der bereits in der Einleitung erwähnten Anordnung[2,3,17] ist in Fig. 20 schematisch, und in Fig. 21 in Gesamtansicht wiedergegeben[20,51]. Der Trennraum ist 30 cm hoch, 20 cm breit und 0.8 cm tief, und mit Glaspulver, Seesand, oder Glasperlen von 0.1 bis 0.5 mm Durchmesser gefüllt. Die Trennkammer wird durch zwei vorne und hinten angebrachte Kühlkammern mit Leitungswasser gekühlt.

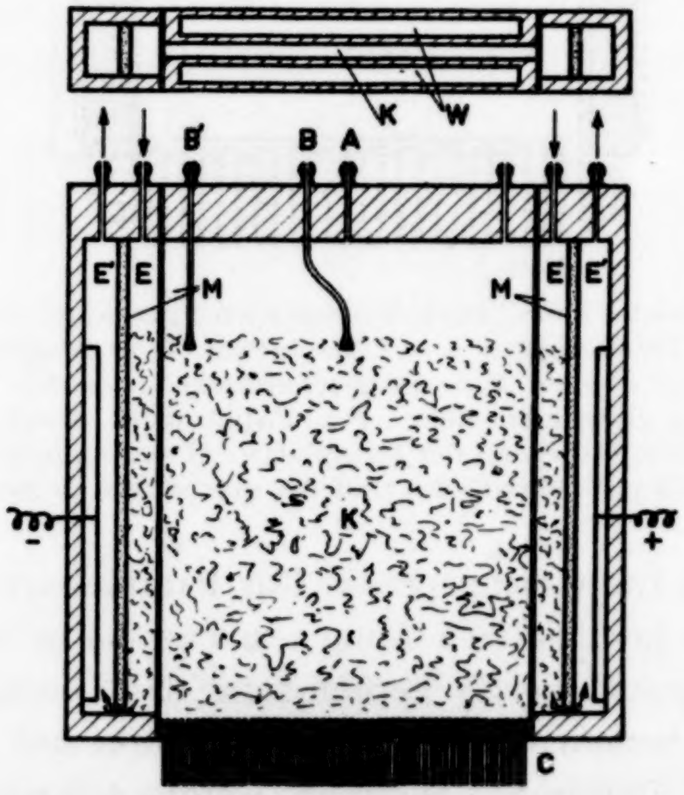


Fig. 20. Schematische Darstellung der Trennküvette nach GRASSMANN UND HANNIG[51]. K = Trennraum; M = Cellophanmembranen oder Glasdiaphragmen; E, E' = Elektrodenräume mit vertikalen Platinelektroden; W = Kühlkammern; A = Zufuhr der Lösung des Grundelektrolyten; B oder wahlweise B' = Zufuhr des zu trennenden Substanzgemisches; C = 50 Röhrchen zur Abfuhr der Flüssigkeit.

Der Trennraum ist von den beiden Elektrodenräumen durch Glasdiaphragmen oder Cellophanmembranen getrennt. Die vertikalen Platinelektroden werden mit der Elektrolytlösung in rascher Strömung gespült. Der Druckausgleich an den beiden

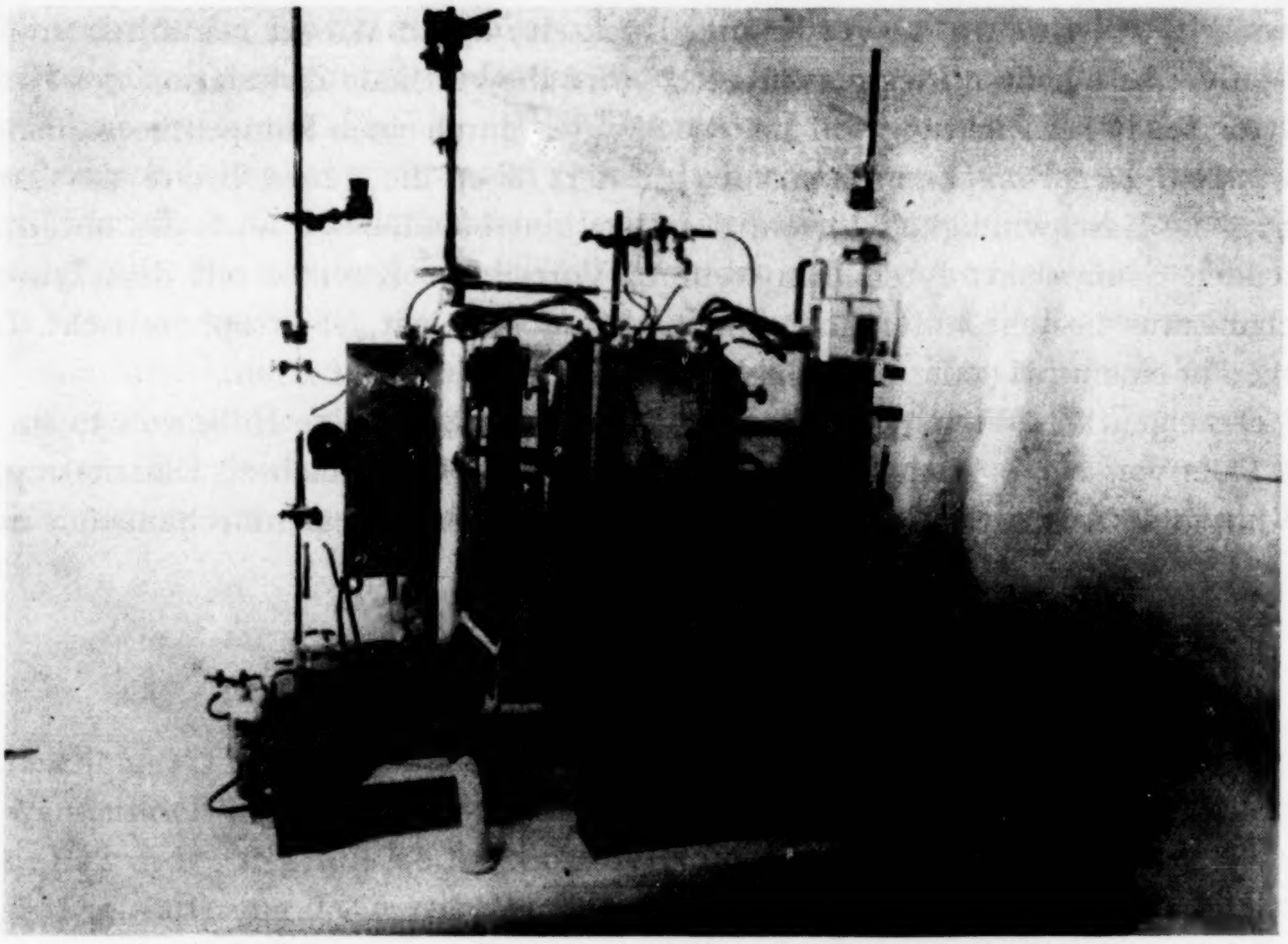


Fig. 21. Gesamtansicht der Trennapparatur nach GRASSMANN UND HANNIG[20].

Seiten der Membranen bzw. der Glasdiaphragmen wird durch Niveaugefässe erreicht. Dieses ist wichtig, da sonst die Flüssigkeit im Trennraum ein seitliches Strömen in die Elektrodenkammern oder umgekehrt aufweisen würde. Durch die Niveaugefässe kann auch die elektroosmotische Strömung im Trennraum kompensiert werden. Entlang des unteren Teiles der Trennkammer sind 50 Röhrchen zur Abfuhr der Flüssigkeit vorgesehen. Es wird eine Gleichspannung zwischen 500 und 1200 V und Stromleistungen bis zu 150 W verwendet. Die Durchlaufszeit des Grundelektrolyten durch die Trennkammer beträgt zwischen 3 und 40 Minuten. Es können noch Stoffe getrennt werden, deren elektrophoretische Beweglichkeiten sich um etwa 10% unterscheiden.

2. *Pumpenapparat nach Brattsten*

(*i*) *Allgemeines.* Als GRASSMANN UND HANNIG[2,3] ihre erste Apparatur entwickelten, wurde gleichzeitig und unabhängig in Schweden an einer sehr ähnlichen Apparatur von SVENSSON UND BRATTSTEN[3] gearbeitet. Diese bestand im wesentlichen aus einer mit Glaspulver sorgfältig gefüllten rechtwinkligen Plexiglasküvette von Dimensionen 20 cm × 32 cm × 2 cm. Die vertikalen Elektrodenräume wurden durch eine perforierte Wand vom eigentlichen Trennraum getrennt. Das Flüssigkeitsniveau und somit der hydrostatische Druck im Inneren des Trennraumes wurden durch Mariottesche Flaschen aufrechterhalten.

Anschliessend hat sich dann BRATTSTEN ausführlich und eingehend mit den apparativen Einzelheiten derartiger Apparaturen befasst, und einen Pumpenapparat entwickelt[52–54]. In einer an Vorder-und Rückseite durch Wasser gekühlten und mit Glaspulver beschickten Plexiglasküvette[54] wird die vertikale Zirkulation des Grundelektrolyten in der Richtung von oben nach unten durch einen Pumpenmechanismus[52] erzwungen. Der Pumpenmechanismus diktiert über die ganze Breite des Trennraumes die Geschwindigkeit sowohl des oben eintretenden wie auch des unten austretenden Grundelektrolyten. Ein weiterer Vorteil der Küvette mit dem Pumpenmechanismus besteht unter anderem in der Möglichkeit, elektrophoretische Trennungen in einem pH- oder Leitfähigkeitsgradienten durchzuführen.

Der eigentliche Trennraum hat eine Breite von 28 cm, eine Höhe von 30 cm und eine Tiefe von 1 cm. Oben sind am Trennraum der Breite nach 28 Eintrittsnippeln und unten 56 Austrittsnippeln angebracht, die mit dem Pumpenmechanismus durch Polythenschläuche verbunden sind. Der Pumpenmechanismus besteht im wesentlichen aus peristaltischen Schlauchpumpen, die zu mehreren in Pumpenblocks vereinigt sind, und durch eine gemeinsame Welle betrieben werden. Die schematische Darstellung eines peristaltischen Pumpenelements sowie sein Funktionieren sind in Fig. 22 wiedergegeben. Die Pumpenblocks werden durch einen gemeinsamen Elektromotor über Kettentrieb betrieben. Die Umlaufgeschwindigkeit der Auslaufpumpen ist gegenüber der der Einlaufpumpen im Verhältnis 1:2 übersetzt, da die Zahl der Auslaufnippeln zweimal so gross ist wie die der Einlaufnippeln. Das Kühlwasser wird durch eine Umlaufpumpe aus einem thermostatierten Kühlwasserbad durch die

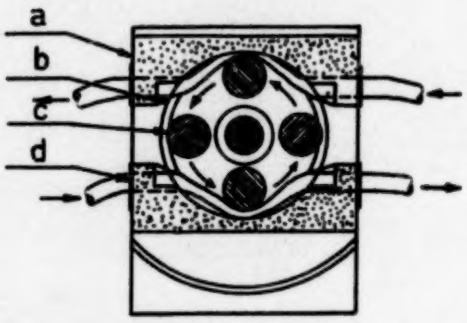


Fig. 22. Schematische Darstellung eines peristaltischen Pumpenelements nach Brattsten[52]. a = Gehäuse; b = rotierende Scheibe; c = Rollen zum Abquetschen der Schläuche d aus weichem Gummi.

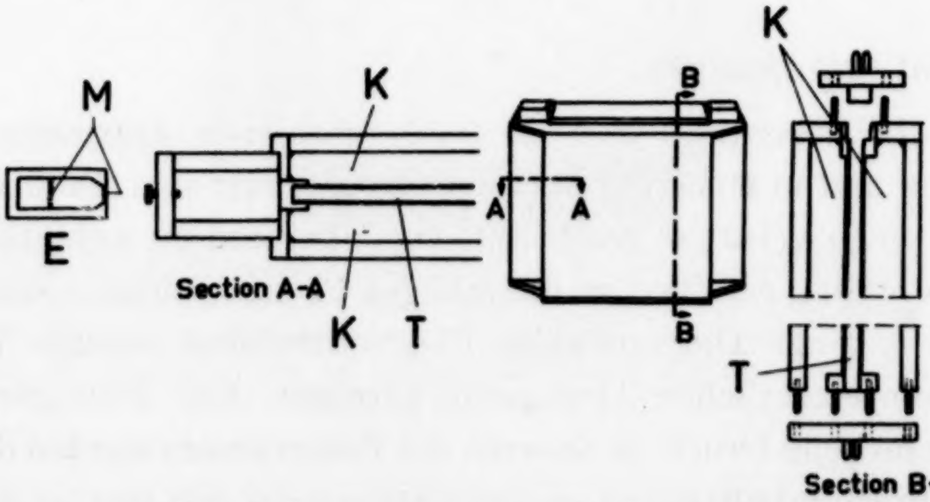


Fig. 23. Die Trennküvette des Pumpenapparates nach Brattsten[54]. Im Schnitt A–A ist die Elektrodenkammer separat links aufgezeichnet. T = Trennraum; K = Kühlkammern; E = Elektrodenkammer; M = Membranen.

Kühlkammern der Küvette zirkuliert. Die Elektrodenkammern werden separat von aussen an die Trennkammer befestigt, und sind vom Trennraum durch Cellophanmembranen getrennt (Fig. 23). Die Elektrodenkammern sind ihrerseits in zwei Räume durch Cellophanmembranen geteilt. In den äusseren Räumen befinden sich in freier Flüssigkeit die Platindrahtelektroden, und die inneren sind mit grobem Glaspulver gefüllt. Die Elektrodenflüssigkeiten werden den Elektroden aus zwei Mariotteschen Flaschen zugeführt, durch Pumpen abgeführt, in einem Gefäss vermischt, und über eine Zirkulationspumpe wieder in die Mariotteschen Flaschen zurückgepumpt.

(*ii*) *Automatische Steuerung der Stromstärke und Beeinflussung der Temperatur im Trennraum.* Bei gleichbleibenden Abmessungen des Trennraumes ist die Austrittstelle einer Fraktion nur durch den Neigungswinkel α ihrer Bahn gegen die Vertikale gegeben.

$$\mathrm{tg}\,\alpha = \frac{v}{W} \tag{1}$$

In Gleichung (1) bedeutet v die horizontale oder elektrophoretische Geschwindigkeit der wandernden Fraktion, und W die vertikale Geschwindigkeit des Grundelektrolyten. Es wurden folgende Voraussetzungen angenommen:

1. Die Änderung der vertikalen Geschwindigkeit ist der Änderung der Umlaufsgeschwindigkeit des Motors des Pumpenapparates proportional.
2. In einem beschränkten Temperaturintervall ist die Änderung der elektrophoretischen Geschwindigkeit proportional der Änderung der Stromstärke im Trennraum, und unabhängig von Temperaturschwankungen.
3. Die Austrittstelle einer Bahn bleibt räumlich und zeitlich konstant, wenn für ein konstantes Verhältnis der Umlaufgeschwindigkeit des Motors des Pumpenapparates und der Stromstärke durch den Trennraum gesorgt wird.
4. Ein Gerät zur Konstanthaltung des Stromes kontrolliert gleichzeitig die Temperatur im Trennraum.

Ein System, das durch Konstanthaltung des Verhältnisses zwischen der Stromstärke im Trennraum und der Umlaufsgeschwindigkeit des Motors des Pumpenapparates automatisch die Stromstärke, und damit auch die Temperatur, im Trennraum reguliert, wurde von LANNEFORS[55] angegeben. Es wurde ein rückgekoppeltes Regelsystem mit magnetischer Verstärkung verwendet. Ein sehr vereinfachtes Schema des Regelsystems ist in Fig. 24 wiedergegeben. Für eine definierte Umlaufgeschwindigkeit des Pumpenmotors v soll die Stromstärke c im Trennraum konstant gehalten werden. Wenn sich z.B. die Stromstärke etwas vergrössert, wächst auch der Spannungsabfall am Wiederstand R_1. Der Pumpenmotor ist mit einem Tachometergenerator direkt gekuppelt. Der Spannungsabfall an R_1 ist gegen die Tachometerspannung r gekuppelt, und es resultiert eine Differenzspannung e, die den magnetischen Verstärker zwingt, seine Ausgangsspannung zu vermindern, um damit den Strom durch den Trennraum auf die ursprüngliche Stärke zu regulieren. Die Genauigkeit der Restauration der Stromstärke wächst proportional der magnetischen Verstärkung.

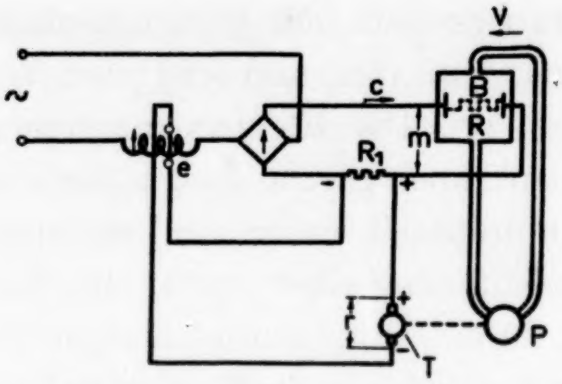


Fig. 24. Vereinfachtes Schema des rückgekoppelten Regelsystems mit magnetischer Verstärkung nach LANNEFORS[55]. B = Trennraum; P = Pumpe; T = Tachometergenerator, dessen Spannung r proportional der Umlaufgeschwindigkeit der Pumpe ist.

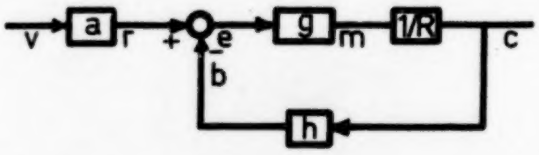


Fig. 25. Schaltschema der Regelungsanordnung nach LANNEFORS[55].

Aus dem Schaltschema in Fig. 25 geht unmittelbar hervor:

$$m = ge \quad (2) \qquad r = av \quad (5)$$

$$e = r - b \quad (3) \qquad c = \frac{1}{R}\, m \quad (6)$$

$$b = hc \quad (4)$$

c = kontrollierte Stromstärke im Trennraum

v = Umlaufsgeschwindigkeit des Motors des Pumpenapparates

r = Tachometerspannung, proportional der Umlaufsgeschwindigkeit v

b = Spannungsabfall am R_1, proportional der kontrollierten Stromstärke

e = Differenzspannung zwischen r und b

m = Klemmspannung an den Elektroden der Trennküvette

R = elektrischer Widerstand des Trennraumes

g = magnetische Verstärkung

h = Proportionalitätsfaktor zwischen b und c ($h = R_1$)

a = Proportionalitätsfaktor zwischen r und v.

Aus (2) bis (6) geht hervor:

$$c = \frac{\frac{g}{R}\, a}{1 + \frac{gh}{R}} \cdot v \qquad (7)$$

Für grosse Verstärkung g gilt: $\frac{gh}{R} \gg 1$

Gleichung (7) kann approximiert geschrieben werden als:

$$c = \frac{a}{h} \cdot v \qquad (8)$$

Gleichung (8) zeigt (da a und h Konstanten sind), dass das Verhältnis zwischen der Stromstärke c im Trennraum und der Umlaufsgeschwindigkeit des Pumpenmotors v konstant ist, und dass die Stromstärke c unabhängig vom Widerstand des Trennraumes ist.

Der Pumpenmotor ist ein 50-Watt Gleichstrommotor, dessen Rotor und Stator in Serie geschaltet sind, und Umlaufgeschwindigkeiten zwischen 2000 und 4000 U. in der Minute gestattet. Er wird über einen variabelen Transformator und Selengleichrichter mit auf 1% stabilisiertem Wechselstrom gespeist. An die Welle des Motors ist der Tachometergenerator gekuppelt.

Die Prüfung der Voraussetzungen (3) und (4) am Beispiel der 46-stündigen Fraktionierung eines normalen menschlichen Serums gibt Fig. 26 wieder. Es wurden 150 ml des 1.8% Protein enthaltenden Serums fraktioniert. Als Grundelektrolyt wurde Veronalpuffer der Ionenstärke 0.03 und pH = 8.6 verwendet. Die Volumgeschwindigkeit war 3.5 ml pro Stunde und cm², die Stromstärke 240 mA, die Leistung 135 W, und die Temperatur des Trennraumes 21°. Über die ganze Versuchsdauer konnte man praktisch keine Schwankungen der Austrittstellen der einzelnen Proteinfraktionen wahrnehmen.

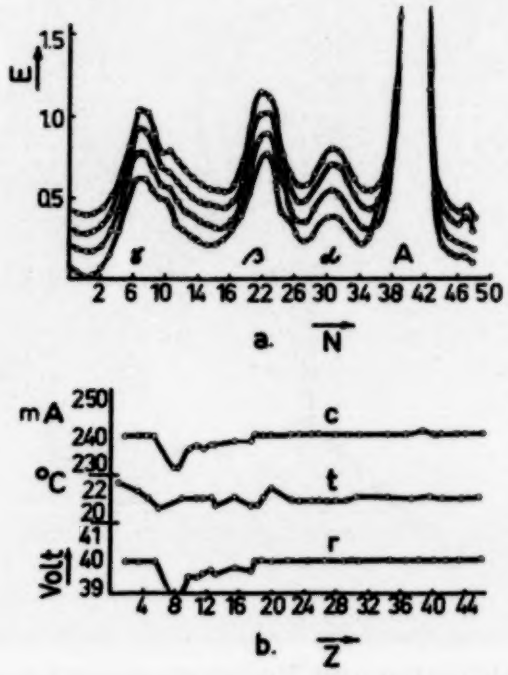


Fig. 26. Fraktionierung eines normalen menschlichen Serums nach BRATTSTEN[53]. (a) Optische Dichte der Fraktionen bei 284 mμ als Funktion der Nummer der Auffanggläschen. Die Skala der optischen Dichte gilt nur für die unterste Kurve. Von den anderen Kurven müssen jeweils 0.1, 0.2 bzw. 0.3 Einheiten subtrahiert werden. N = Nummer des Auffangglases; E = optische Dichte; A = Albumine; α = α-Globuline; β = β-Globuline; γ = γ-Globuline. (b) r = Generatorspannung; c = Stromstärke im Trennraum; t = Temperatur des Trennraumes; Z = Zeit.

Zum Abschluss wird noch eine Gesamtansicht von Trennküvette und Pumpenapparat neuester Bauart nach BRATTSTEN[56] in Fig. 27 wiedergegeben. Das Gerät entspricht was Küvette, Regulationsapparat, Kühlung und Pumpenapparat anbetrifft, im wesentlichen der bereits beschriebenen Anordnung.

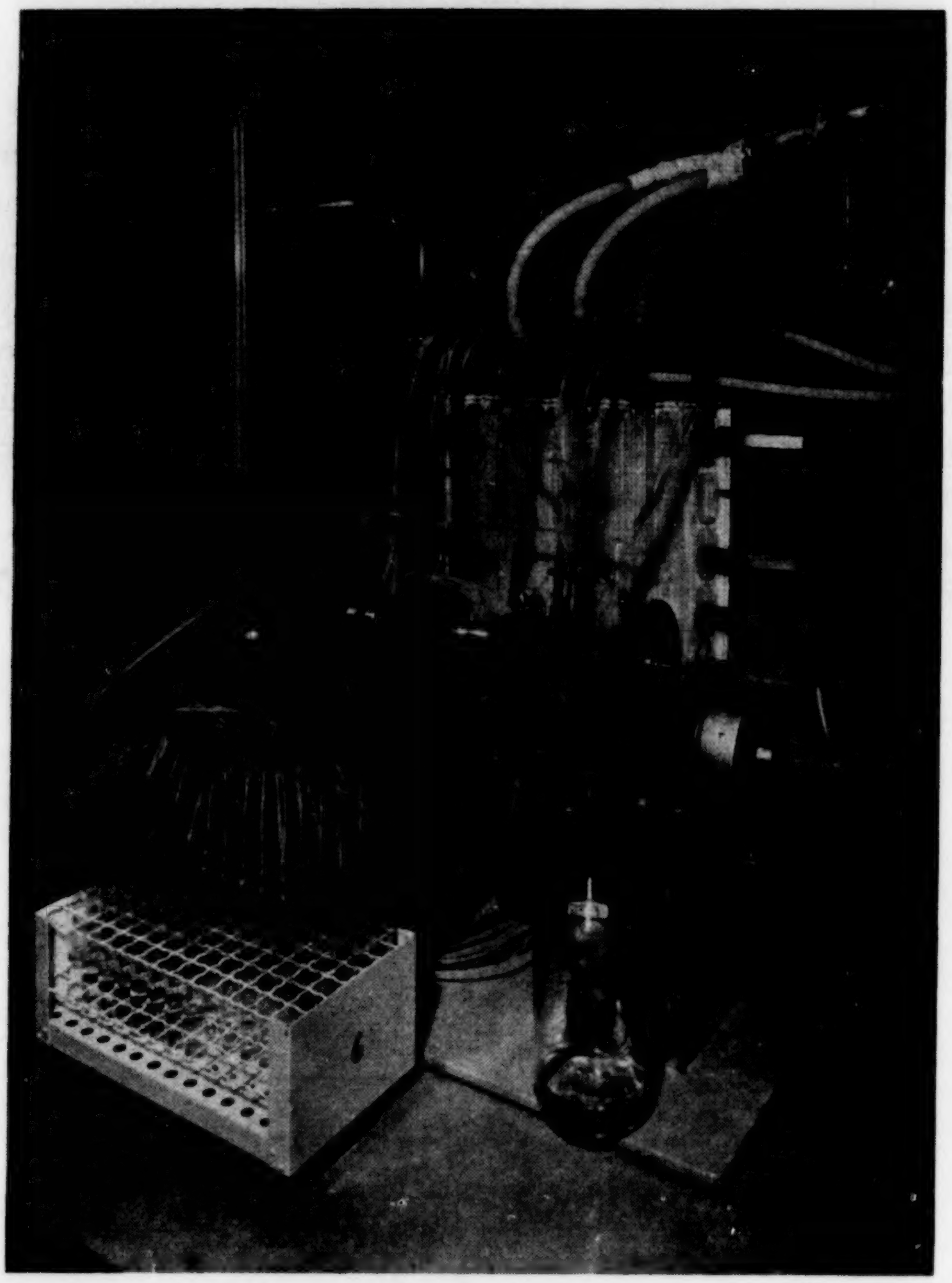


Fig. 27. Gesamtansicht an Trennküvette und Pumpenapparat neuester Bauart nach BRATTSTEN[56]. (Hersteller: Fa. ESAB, Laxa, Schweden.)

(c) *Freie kontinuierliche Elektrophorese*

1. *Apparatur mit aufwärts gerichteter forcierter Laminarströmung in einem Verdickungsmittel nach Dobry und Finn*

In der Bestrebung, die kontinuierliche elektrophoretische Trennung vom Laboratoriumsmasstab, d.i. Durchsatz in der Grössenordnung ml/Stunde, in einen technischen Masstab zu überführen, d.i. Durchsatz in der Grössenordnung l/Stunde, wurde von DOBRY UND FINN[57, 58] die in Fig. 28 schematisch wiedergegeben Anordnung vorgeschlagen. Die Trennung findet in einer Kolonne aus Plexiglas statt, deren Masse *ca.* 130 cm × 8 cm × 5.5 cm betragen. Die Kolonne hat einen rechteckigen Querschnitt und ist mit einer viskosen Grundelektrolytlösung gefüllt, die sich in einer aufwärts gerichteten laminaren Strömung befindet. Dem Grundelektrolyt wird Methocel, Polyvinylalkohol oder Dextran zugegeben, um die erforderliche Viskosität der Lösung (*ca.* 10 cp) zu erreichen. Das zu trennende Gemisch wird von unten durch eine spaltförmige Düse (40 × 2 mm) in die Kolonne kontinuierlich eingeführt. Oben, am Ausgang der Kolonne, sind mehrere spaltförmige Auslaufdüsen (45 × 3 mm) angebracht. Der elektrische Strom fliesst senkrecht zur Strömungsrichtung der viskosen Flüssigkeit. Die in der Richtung von unten nach oben bespülten Elektroden befinden sich in den Elektrodenkammern, die durch Membranen aus thermoplastischem Harz von der Trennkolonne getrennt sind.

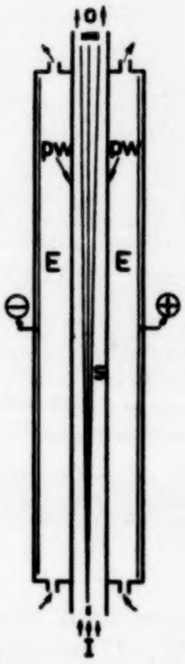


Fig. 28. Schematische Darstellung der Apparatur mit aufwärts gerichteter forcierter Laminarströmung in einem Verdickungsmittel nach Dobry und Finn[57,58]. S = Trennkolonne mit der viskosen Flüssigkeit die sich in aufwärts gerichteter forcierter Laminarströmung befindet; I = spaltförmige Einlaufdüse; O = spaltförmige Auslaufdüsen; E = Elektrodenkammern; PW = Membranen aus einem thermoplastischen Harz.

Beim Ausschalten des Stromes fliesst das zu trennende Gemisch in einem einzigen bandförmigen laminaren Strom aus der Einlaufdüse nach oben gegen die mittlere Auslaufdüse. Beim Einschalten des Stromes wird das Band in mehrere Bänder aufgespalten, die dann über entsprechende Auslaufdüsen einzeln gesammelt werden können. Die Kapazität des Apparates beträgt z.Z. *ca.* 9 ml pro Minute der zu trennenden Substanzlösung, und zwar bei der Geschwindigkeit des Grundelektrolyten von 0.5 cm pro Sekunde. Bis jetzt sind nur Farbstoffmischungen auf diese Weise getrennt worden. Für die Trennung der Serumproteine muss die Apparatur noch erheblich verbessert werden, wegen zehnmal kleinerer Beweglichkeiten der Proteinfraktionen. Es müssen noch eingehend die Bedingungen studiert werden, unter welchen die Bänder gleichmässig, ungestört und reproduzierbar verlaufen. Eine ganze Reihe sehr wichtiger Variablen, wie z.B. Strömungsgeschwindigkeit, Viskosität, Konzentration der Pufferlösung und Höhe der Trennkolonne wurden bisher noch nicht genügend studiert. Die zukünftigen Verbesserungen könnten vielleicht auch zu einer rationellen Trennung von Enzymen und Serumproteinen in grossem Masstab führen.

2. *Elektrophorese in einer dünnen Flüssigkeitsschicht zwischen zwei planparallel aufgestellten Platten*

(*i*) *Elektrophorese im Dichtegradienten nach Philpot.* Bereits im Jahre 1939, 10 Jahre vor der Veröffentlichung des Patentes von GRASSMANN[2], hat PHILPOT[59] eine kontinuierliche elektrophoretische Trennung in dünnen Flüssigkeitsschichten vorgeschlagen, die gegenüber der diskontinuierlichen Trennung im U-Rohr erhebliche Vorzüge für präparative Trennungen von Proteinen haben sollte. Die Anordnung, so wie sie 1939 der Faraday Society zur Diskussion über die elektrische Doppelschicht verabreicht wurde, ist in Fig. 29 wiedergegeben.

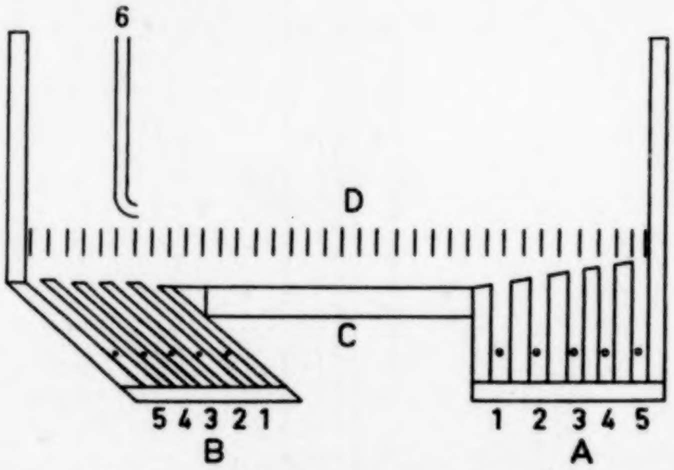


Fig. 29. Schematische Wiedergabe der Apparatur nach PHILPOT[59]. A = Eingangschlitze (1–5); B = Ausgangschlitze (1–5); 6 = Saugrohr; C = Anode aus Kohlenstoff; D = Kathode aus Kupferfolien.

Diese Anordnung soll hier nur kurz besprochen werden. Zwischen zwei Platten aus Plexiglas strömt die Flüssigkeit in horizontalen Schichten, die durch wachsende Dichte in der Richtung von oben nach unten stabilisiert sind, von den Eingangsschlitzen A (1–5) zu den Ausgangsschlitzen B (1–5), d.h. in der Richtung von rechts nach links. Die Anode aus Kohlenstoff C befindet sich unten, und wird durch die erste Flüssigkeitsschicht (grösste Dichte) die aus 5 M Ammoniumbromid besteht, unter Bromentwicklung, gespült. Die Schichten 2, 3 und 4 enthalten eine Pufferlösung von 0.1 M Ammoniumchlorid und 0.005 M Ammoniak. Die Flüssigkeitsschichte No. 2 enthält zur Erhöhung der Dichte 4% Glycerin, die Schichte No. 3 und 4.11% Aethylalkohol. Die Schichte No. 3 enthält ausserdem noch 1.5% des zu trennenden Proteingemisches. Schicht No. 5 enthält 0.5 M Ammoniumchlorid, 0.5 M Salzsäure und 40% Methylakohol. Die Kathode D besteht aus einer vertikalen Reihe von Kupferfolien. Die Proteinmischung wird im vertikalen elektrischen Feld in Fraktionen auseinandergespalten, und verlässt durch die Ausgangsschlitze (2–4), wo sie durch eine Schlierenanordnung beobachtet wird, die Apparatur. Die zulässige Stromstärke ist 6 A und sollte, bei entsprechender Vergrösserung der Apparatur in zwei Dimensionen, auf 100 A vergrössert werden.

(*ii*) *Trägerfreie Durchflusselektrophoreseapparatur nach Barrollier, Watzke und Gibian.* Eine Apparatur die ohne jeder Trägersubstanz und sogar ohne Verwendung von Verdickungsmitteln arbeitet, haben neulich BARROLLIER, WATZKE UND GIBIAN[60] beschrieben (Fig. 30).

Die Pufferlösung fliesst langsam in Form einer *ca.* 0.5 mm dicken Schicht zwischen zwei gekühlten nahezu horizontal liegenden Glasplatten. Die Grösse der Glasplatten ist *ca.* 60 cm × 60 cm. Die Pufferlösung wird von hinten über einen Zellstoffstreifen zugeführt, der mittels 7 aus einer Mariotteschen Flasche gespeister Kanülen aus rostfreiem Stahl betropft wird. Der elektrische Strom fliesst quer zur Pufferrichtung. Der elektrische Kontakt wird über Filtrierpapierstreifen vermittelt, die in seitliche Elektrodentröge eintauchen. Zum Abtropfen des Puffers an der vorderen Kante der Platten dienen 56 schmale Streifen aus Zellstoff, die kurz in den Flüssigkeitsspalt hineinragen. Alle diese Streifen halten zugleich die obere und die untere Glasplatte im gewünschten Abstand. Die Elektrodentröge werden mit der Pufferlösung gespült, die aus einer 25-l Kunststofflasche mittels einer Membranpumpe unter Tropfunterbrechung gefördert wird. Eine peristaltische Schlauchpumpe fördert das zu untersuchende Gut über eine Glaskapillare in eine der Bohrungen an der oberen Platte. Mit einem verstellbaren Schubrad kann die Pumpenleistung zwischen 0.5 und 8.8 ml/Std. kontinuierlich eingestellt werden. Die Durchlaufszeit für den Pufferfilm beträgt zwischen 45 und 60 Min. Die Kältemaschine mit dem Umlaufthermostaten zur Temperatur-Konstanthaltung der umlaufenden Kühlflüssigkeit hat eine Leistung von 1500 kcal/Std. Die Pumpenleistung beträgt 6 l/Min. Als Stromquelle dient ein elektromagnetischer Spannungskonstanthalter, der kontinuierlich von 500 bis 3000 V regelbar ist. Die maximale Strombelastung beträgt 500 mA.

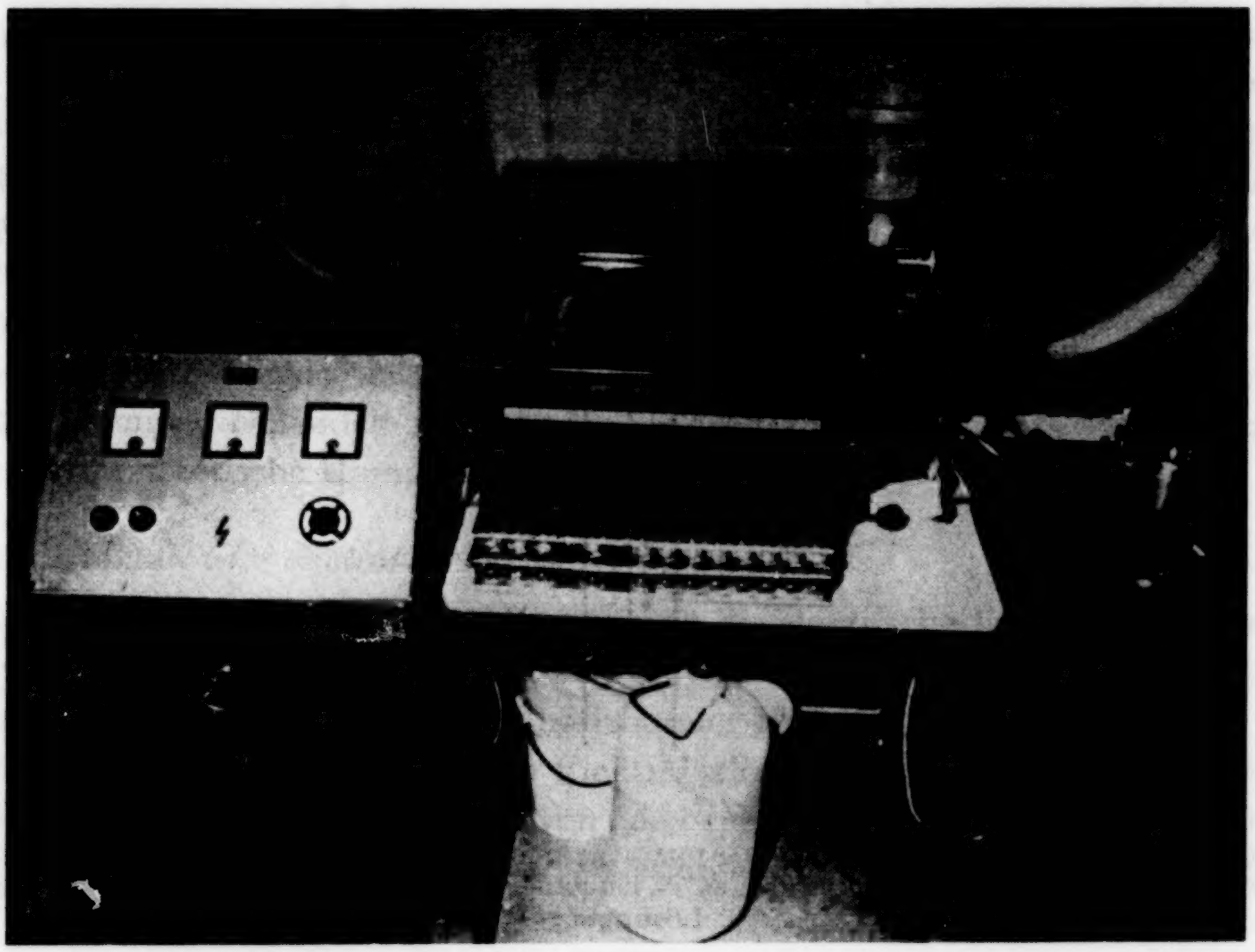


Fig. 30. Gesamtansicht der trägerfreien Durchfluss-Elektrophoreseapparatur "Pheroplan"[61] nach BARROLLIER, WATZKE UND GIBIAN[60].

3. *Kontinuierliche Elektrophorese-Konvektion-Apparatur nach Bier*

Eine Apparatur die, wie die bereits beschriebene, auch ohne Verdickungsmittel arbeitet, und eine relativ grosse Trennkapazität von 4 ml/Min. aufweist, wurde von Bier[62] angegeben. Diese Apparatur beruht auf dem Prinzip der Elektrodekantation von Pauli[63], und infolgedessen gestattet nur Trennungen von hochmolekularen Ampholyten. In einem Arbeitsgang kann jeweils nur eine Komponente aus dem Gemisch abgetrennt werden. Die Apparatur besteht im wesentlichen (Fig. 31) aus zwei äusseren semipermeablen Membranen, die parallel in einem Abstand von 3–4 mm angebracht sind, und einer dritten semipermeablen Membrane, die sich in der Mitte zwischen den beiden Membranen befindet, aber nicht ganz bis zum Boden der Anlage reicht. Damit ist die Zelle in zwei obere und einen gemeinsamen unteren Raum geteilt, von denen jeder mit einer Vorrichtung für kontinuierliche Zufuhr bzw. Abfuhr der kolloiden Lösungen versehen ist. Das Ganze taucht in einer gekühlten zirkulierenden Pufferlösung, und wird senkrecht zur Membranebene vom elektrischen Strom durchflossen. Die Stromstärke beträgt 2 A, und es wurden Membranen mit den Flächen von 13 bis 125 cm^2 verwendet. Unter Konstanthaltung der Stromstärke wird der Trenneffekt wenig durch Änderung der Membranflächen beeinflusst.

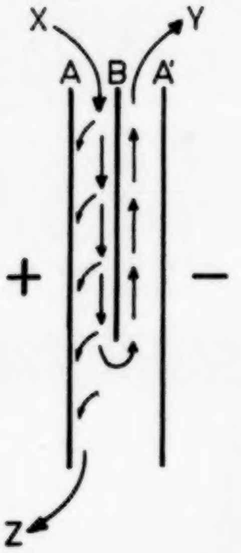


Fig. 31. Die Elektrophorese-Konvektion-Apparatur nach Bier[62] (schematisch). A, A′ = äussere Membranen; B = Zwischenmembrane; X = Eingang der Lösungen; Y, Z = Ausgang der Lösungen.

Zur Trennung eines Proteingemisches wird das pH der Pufferlösung so gewählt, dass eine der Komponenten isoelektrisch vorliegt, und alle anderen Komponenten Ladungen mit gleichen Vorzeichen aufweisen. Die Stromrichtung wird so gewählt, dass die geladenen Komponenten gegen die äussere Membran wandern, dort angereichert werden, infolge grösserer Dichte zu Boden fallen, und dort kontinuierlich abgeführt werden. Die isoelektrische Komponente wird durch den elektrischen Strom nicht beeinflusst; sie wird durch die Flüssigkeitsströmung bei Y abtransportiert. Nur diese Komponente kann rein erhalten werden, da die elektrisch wandernden unten abtransportierten Komponenten die isoelektrische Komponente in ursprünglicher Konzentration enthalten.

Für diskontinuierliche Trennungen wurde dieses Prinzip bereits im Jahre 1920 von **Samec**[64–66], 1941 von **Kirkwood**[67] und 1943 von **Gutfreund**[68] verwendet. Eine quantitative Theorie der Konvektionselektrophorese haben **Kirkwood** und Mitarb.[69,70] 1950 angegeben.

3. ALLGEMEINES

(*a*) *Der Prozess in feuchter Kammer*

Die Bewegung eines elektrisch geladenen Teilchens, das reversibel vom Träger (Trennbogen, usw.) adsorbiert wird, setzt sich nach **Pučar**[36] bei der kontinuierlichen Elektrophorese als vektorische Summe der horizontalen oder elektrochromatographischen und vertikalen oder chromatographischen Komponente zusammen. Wegen Vereinfachung soll zunächst die horizontale Geschwindigkeitskomponente für ein unadsorbierbares Teilchen unter Bedingungen der kontinuierlichen Elektrophorese in feuchter Kammer behandelt werden. Die horizontale Komponente kann dann als reine elektrophoretische Komponente beschrieben werden.

1. *Die horizontale oder elektrophoretische Komponente*[36]

Bei der Elektrophorese in feuchter Kammer ist die elektrische Feldstärke wegen der Verdunstung des Lösungsmittels, bzw. Konzentration des Elektrolyts, und der Kapillarsaugwirkung des Filtrierpapiers in horizontaler Richtung nicht konstant[71,72].

Im stationären Zustand wird die Elektrolytlösung sowohl aus der Pufferwanne (W) in der Vertikalrichtung, wie auch von den beiden Elektrodenrinnen (E) her in der Horizontalrichtung vom Filtrierpapier angesaugt. Fig. 32 schildert schematisch den Verlauf der elektrischen Feldstärke (*H*-Kurven) am Filtrierpapier im stationären Zustand und in vier charakteristischen Horizontalen.

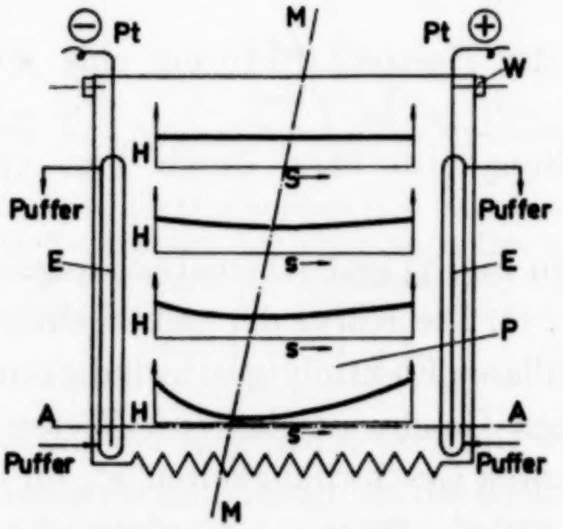


Fig. 32. Schematischer Verlauf der elektrischen Feldstärke (*H*-Kurven) im Stationärzustand am Filtrierpapier[36]. Die Krümmung der *H*-Kurven wächst im Stationärzustand infolge Verdunstung und Kapillarwirkung von oben nach unten. P = Filtrierpapier; W = Pufferwanne; E = Elektrodenrinne mit Platinelektrode; M–M = Lage der Minima der *H*-Kurven, bzw. der maximalen Elektrolytkonzentration in der betreffenden Horizontale; A–A = eine charakteristische Horizontale.

Im folgenden wird eine anodische elektrophoretische Bewegung der elektrisch geladenen Teilchen betrachtet. Eine kathodische Bewegung würde prinzipiell der anodischen Bewegung gleichen. Dabei soll davon abgesehen werden, dass die Beweglichkeit der Teilchen (u), wegen des Durchlaufens von Konzentrationsgradienten im Elektrolyt und der dadurch bedingten Änderung des ζ Potentials, nicht streng konstant ist.

Die apparente momentane Geschwindigkeit eines Teilchens mit der Beweglichkeit u im elektrischen Feld H ist[71]:

$$v' = uH - w' \tag{9}$$

Hier bedeutet w' die Summe der kapillaren Elektrolytgeschwindigkeit in der Horizontalrichtung und der elektroosmotischen Geschwindigkeit. Die Differenzierung nach s ergibt:

$$\frac{dv'}{ds} = u\frac{dH}{ds} - \frac{dw'}{ds} \tag{10}$$

Daraus folgt für

$$\text{(a)} \quad u\frac{dH}{ds} = \frac{dw'}{ds}, \qquad \frac{dv'}{ds} = 0, \qquad v' = \text{Konst.} \tag{11}$$

Das Teilchen bewegt sich in der Horizontalrichtung mit konstanter Geschwindigkeit.

$$\text{(b)} \quad u\frac{dH}{ds} < \frac{dw'}{ds}, \qquad \frac{dv'}{ds} < 0 \tag{12}$$

Das Teilchen erfährt in der Horizontalrichtung eine apparente Beschleunigung der Bewegung.

$$\text{(c)} \quad u\frac{dH}{ds} > \frac{dw'}{ds}, \qquad \frac{dv'}{ds} > 0 \tag{13}$$

Das Teilchen erfährt in der Horizontalrichtung eine apparente Verzögerung der Bewegung.

Die Differenzialgleichung (10) kann durch Fig. 33 einfach veranschaulicht werden.

Die obere Zeichnung in Fig. 33 gibt schematisch folgende Kurven in der Horizontale A–A (Fig. 32) wieder: (1) Die Kurve der elektrischen Feldstärke H, und (2) die Kurve der Summe der kapillaren Elektrolytgeschwindigkeit in der Horizontalrichtung und der elektroosmotischen Geschwindigkeit w'. Weiter sind nach Gleichung (9) die Kurven für die apparenten Geschwindigkeiten, v'_1 für die Beweglichkeit der Teilchen $u = 1$, v'_2 für $u = 2$, und $v'_{0.5}$ für $u = 0.5$, aufgezeichnet. Einfachheitshalber sind in Fig. 33 rechts von der M–M Linie die Gradienten dH/ds und dw'/ds gleich gross gezeichnet ($dH/ds = dw'/ds$). Die M–M Linie gibt die Lage der Minima der H-Kurven wieder.

Das Teilchen mit $u = 2$ erfährt rechts von der M–M Linie stets eine apparente Beschleunigung in der Horizontalrichtung, das Teilchen mit $u = 1$ bewegt sich in demselben Bereich mit konstanter Geschwindigkeit, während das Teilchen mit $u = 0.5$ eine Verzögerung der Bewegung erfährt.

Da vorläufig angenommen wird, dass sich keines der drei Teilchen am Filtrierpapier adsorbiert, ist die vertikale Komponente für alle drei Teilchen gleich der vertikalen Elektrolytgeschwindigkeit W. Die untere Zeichnung (Fig. 33) gibt schematisch die Bahnen der Teilchen am Filtrierpapier wieder. Links von der M–M Linie erfahren alle drei Teilchen apparente Verzögerung in der Horizontalrichtung, und wenn der Startpunkt vielleicht noch mehr nach links verschoben würde, könnten die Teilchen in S-förmigen Bahnen über das Filtrierpapier laufen. Obwohl ein kleiner Teil der Bahnen in Fig. 33 links von der M–M Linie verläuft, wurden die kleinen Verzögerungen am Anfang der Bahnen vernachlässigt, da die Krümmungen und die Wirkung der H- und w'-Kurven am oberen Teil des Papiers (am Anfang des Prozesses) klein sind. Die Neigung der M–M Linie zur Vertikale ist durch Elektroosmose bedingt.

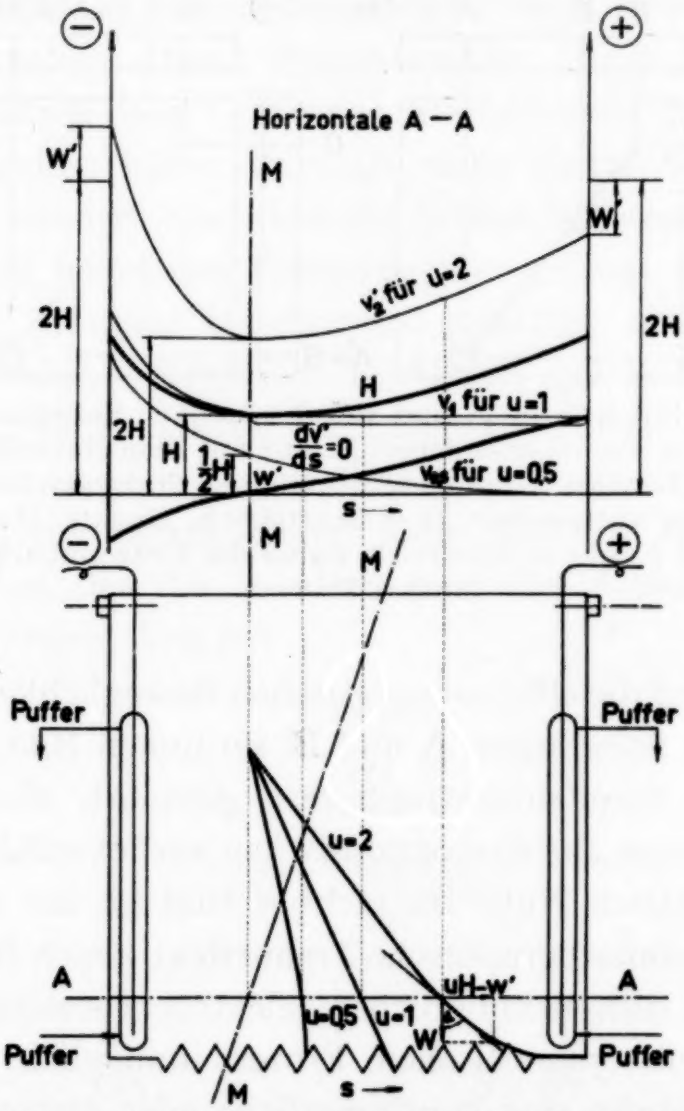


Fig. 33. Oben: die H-und w'-Kurve in der Horizontale A–A im stationären Zustand (schematisch)[36]. Nach Gleichung (9) sind dann die Kurven der apparenten Horizontalgeschwindigkeiten v'_1 für $u = 1$, v'_2 für $u = 2$, und $v'_{0.5}$ für $u = 0.5$ in der Horizontale A–A aufgezeichnet. Unten: schematische Wiedergabe der Bahnen der Teilchen am Filtrierpapier mit den Beweglichkeiten $u = 0.5$, $u = 1$, und $u = 2$.

Neulich haben Peeters, Vuylsteke und Noë[37] gefunden, dass sich bei der kontinuierlichen Elektrophorese in feuchter Kammer nach Erreichung des Gleichgewichtszustandes am Trennbogen eine charakteristische Verteilung der Ionen des Grundelektrolyten einstellt.

2. *Die vertikale oder chromatographische Komponente*[36]

Strain und Sullivan[7] haben schon bemerkt, dass im kontinuierlichen Prozess die Trennung von zwei Substanzen unmöglich ist, wenn das relative Verhältnis ihrer

Elektrowanderung und ihrer chromatographischen Wanderung dasselbe ist. Im diskontinuierlichen Prozess ist die Trennung von zwei Substanzen nur dann unmöglich, wenn ihre Elektrowanderung und gleichzeitig ihre chromatographische Wanderung identisch sind. Fig. 34 soll das veranschaulichen.

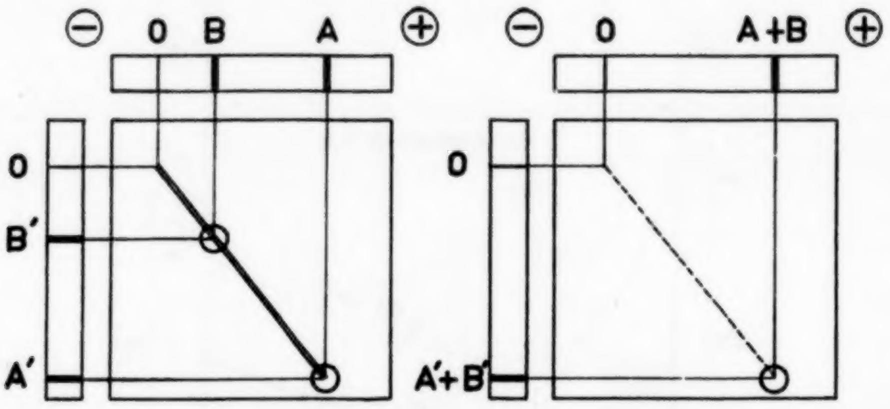


Fig. 34. Die Trennung der Substanzen A und B[36]. Links: Die Substanzen sind im diskontinuierlichen Prozess trennbar. Die Trennbarkeit beruht nur auf dem chromatographischen Effekt. Die elektrophoretischen Beweglichkeiten sind identisch, und deswegen sind die beiden Substanzen im kontinuierlichen Prozess untrennbar. O = Startstrich. Rechts: Der chromatographische wie auch der elektrophoretische Effekt ist identisch, daher die Untrennbarkeit auch im diskontinuierlichen Prozess.

In beiden Fällen sind die elektrophoretischen Beweglichkeiten (*u*) der Substanzen A und B identisch. Die Substanzen A und B, im linken Bild, werden zwar sehr gut durch eindimensionale Streifenelektrophorese getrennt, aber der Trenneffekt ist offensichtlich nur auf einen chromatographischen zurückzuführen. Der elektrophoretische Trenneffekt ist gleich Null. Im rechten Bild ist der elektrophoretische und gleichzeitig auch der chromatographische Trenneffekt gleich Null, und daher ist auch die Trennung in einem diskontinuierlichen (elektrochromatographischen), wie auch im kontinuierlichen (elektrophoretischen) Prozess unmöglich.

Fig. 35 veranschaulicht eine kontinuierliche oder diskontinuierliche Trennung. Die Substanz B wird vom Filtrierpapier adsorbiert, die Substanz A wird im Gegenteil nicht adsorbiert, sondern sie wandert in der Vertikale mit der Flüssigkeitsfront, d.h. mit der Geschwindigkeit *W*.

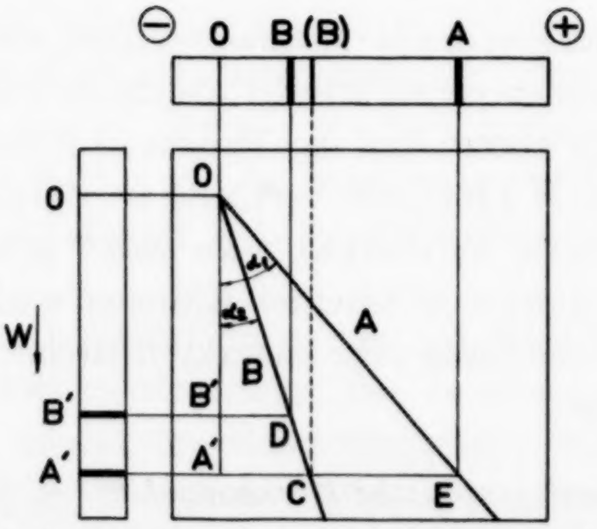


Fig. 35. Die Trennung der Substanzen A und B die verschiedene chromatographische und auch elektrophoretische Eigenschaften aufweisen[36]. Die Trennung ist sowohl im kontinuierlichen wie auch im diskontinuierlichen Prozess möglich. Weitere Erläuterungen im Text.

Die zurückgelegten Wege $\overline{\mathrm{OA}}$, $\overline{\mathrm{OB}}$, $\overline{\mathrm{OA'}}$ und $\overline{\mathrm{OB'}}$ entsprechen einfachheitshalber einem System ohne Verdunstung. Die Länge $\overline{\mathrm{OB}}$ ist dann der apparente elektrophoretische Weg der Substanz B, die übrigen Längen die wahren elektrophoretischen bzw. chromatographischen Wege. In der Zeit, in der die Substanz A den Weg $\overline{\mathrm{OE}}$ zurückgelegt hat, ist die Substanz B, wegen Adsorption, von O nur bis zum Punkt D angelangt. Wenn die Substanz B nicht adsorbiert würde, müsste sie in derselben Zeit bis zum Punkt C vorrücken. Durch Projektion der Länge $\overline{\mathrm{OC}}$ auf die Horizontale bekommen wir den wahren Weg $\overline{\mathrm{O(B)}}$, den die Substanz B mittels Papierstreifenelektrophorese zurückgelegt hätte, wäre sie nicht durch Adsorption vom Filtrierpapier zurückgehalten worden. Nachdem die beiden Substanzen im kontinuierlichen Prozess an den unteren Rand des Filtrierpapiers gelangt sind, ist im stationären Zustand die Bahn der Substanz B adsorptiv gesättigt, und die neuankommenden Stoffmengen erleiden keine Adsorption. Die Bahn der Substanz B wird dadurch keinenfalls geändert, da die Projektion der Länge $\overline{\mathrm{OC}}$ auf die Horizontale wie auch auf die Vertikale den zurückgelegten Wegen ohne Adsorption entspricht.

Die elektrophoretische Beweglichkeit kann aus der Messung des apparenten Weges berechnet werden, den die Substanz in einem Adsorbens zurückgelegt hat. Für ein System ohne Verdunstung gilt:

$$u = \frac{\overline{\mathrm{O(B)}}}{H\tau} \tag{14}$$

$$\overline{\mathrm{O(B)}} = \frac{\overline{\mathrm{OA'}}}{\overline{\mathrm{OB'}}}\,\overline{\mathrm{OB}} \tag{15}$$

und für:

$$\frac{\overline{\mathrm{OB'}}}{\overline{\mathrm{OA'}}} = R_F \qquad u = \frac{1}{R_F}\,\frac{\overline{\mathrm{OB}}}{H\tau} \tag{16}$$

oder

$$u = \frac{u'}{R_F} \tag{17}$$

Hier bedeutet u die wahre Beweglichkeit, u' die apparente Beweglichkeit im Adsorbens, τ die Zeit und R_F ist der bekannte chromatographische Faktor. Nach (17) ist die horizontale Komponente:

$$v' = R_F v \tag{18}$$

bzw.

$$v' = R_F u H \tag{19}$$

und die vertikale Komponente:

$$W' = R_F W \tag{20}$$

und

$$\operatorname{tg}\alpha = \frac{v'}{W'} = \frac{uH}{W}$$

schliesslich:

$$\frac{\operatorname{tg}\alpha_1}{\operatorname{tg}\alpha_2} = \frac{u_1}{u_2} \tag{21}$$

Für den Prozess in feuchter Kammer ist:

$$\mathrm{tg}\,\alpha = \frac{uH - w'}{W}$$

und für denselben Punkt ($w'_1 = w'_2$):

$$\mathrm{tg}\,\alpha_1 - \mathrm{tg}\,\alpha_2 = \frac{H}{W}(u_1 - u_2) \qquad (22)$$

Die Neigungen der Bahnen der Teilchen gegen die Vertikale, im System ohne Verdunstung, verhalten sich wie ihre elektrophoretischen Beweglichkeiten (21). Bei der Elektrophorese in feuchter Kammer sind die Differenzen der Neigungen proportional den Differenzen der Beweglichkeiten, bezogen auf einen identischen Punkt ($w'_1 = w'_2$) (22). Für das System ohne Verdunstung gilt dasselbe, aber jetzt für alle Punkte am Filtrierpapier, weil $w' = 0$. Im kontinuierlichen Prozess sind zwei Substanzen nur dann trennbar, wenn sie verschiedene elektrophoretische Beweglichkeiten besitzen. Eine eventuelle Adsorption hat auf die Trennung keinen Einfluss. Mit anderen Worten: alle Teilchen, die sich auf derselben Bahn befinden, haben identische elektrophoretische Beweglichkeiten.

Jeder diskontinuierliche Prozess ist im wesentlichen ein elektrochromatographischer Prozess. Untrennbar sind nur Substanzen, die gleichzeitig identische elektrophoretische Beweglichkeiten und R_F Werte haben. Bei eindimensionaler Streifenelektrophorese bzw. eindimensionaler Elektrochromatographie sind jene Substanze untrennbar, welche der Gleichung (23) genügen:

$$u_1 R_{F_1} = u_2 R_{F_2} \qquad (23)$$

Tabelle I gibt eine Übersicht über elektrophoretische und elektrochromatographische Prozesse, sowie über die Bedingungen für die Untrennbarkeit.

TABELLE I

ÜBERSICHT ÜBER ELEKTROPHORETISCHE UND ELEKTROCHROMATOGRAPHISCHE PROZESSE, NACH PUČAR[36]

Prozess	*Bedingungen für die Untrennbarkeit*
Kontinuierliche Elektrophorese	$u_1 = u_2$
Zweidimensionale Elektrochromatographie oder zweidimensionale Elektrochromatophorese. (Diskontinuierliche Elektrophorese) (Die Bedingung für diesen Prozess ist die gleichzeitige Trennung in der chromatographischen und elektrophoretischen Richtung)	$u_1 = u_2$ und gleichzeitig $R_{F_1} = R_{F_2}$
Eindimensionale Elektrochromatographie oder eindimensionale Elektrochromatophorese	$u_1 = u_2$ und gleichzeitig $R_{F_1} = R_{F_2}$ oder $u_1 R_{F_1} = u_2 R_{F_2}$
(Eindimensionale) Streifenelektrophorese (R_F-Werte aller Fraktionen sind 1)	$u_1 = u_2$

Eine Illustration der soeben besprochenen Verhältnisse gibt Fig. 36 wieder. Es wurde eine Farbstoffmischung kontinuierlich elektrophoretisch (links), und unter gleichen Bedingungen diskontinuierlich, bzw. zweidimensional elektrochromatographisch (rechts) getrennt. Die Farbstoffmischung bestand aus folgenden Komponenten:

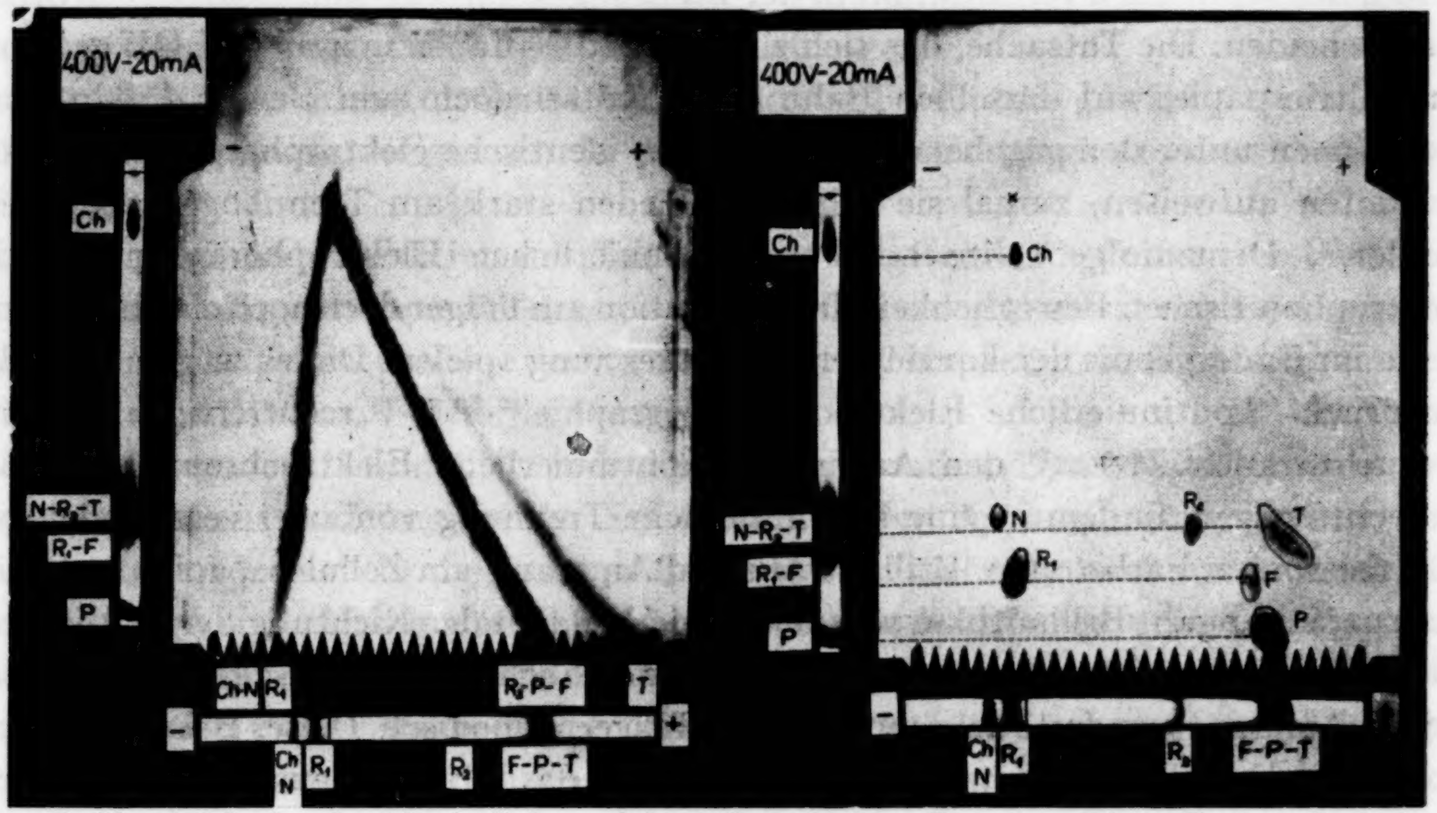


Fig. 36. Vergleich der kontinuierlichen elektrophoretischen Trennung einer Farbstoffmischung (links) mit der, diskontinuierlichen, zweidimensionalen elektrochromatographischen Trennung derselben (rechts) unter untereinander identischen Bedingungen nach PUČAR[36]. Tropäolin O (T), Fluorescein (F), Chlorphenolrot (P), Rosolsäure (R_1 und R_2), Chrysoidin Y (Ch) und Nitranilin (N) in einer Boratpufferlösung von pH = 8.6 und Ionenstärke 0.012. Filtrierpapier Munktell No. 20/150.

Tropäolin O (T), Fluorescein wasserlöslich (F), Chlorphenolrot (P), Rosolsäure (R), Chrysoidin Y (Ch) und Nitranilin (N). Die Konzentration jedes der sechs Farbstoffe in 50%-iger ethanolischer Lösung war 4 mg/ml.

Als Elektrolyt wurde eine Borsäure-Na-Acetat-NaOH Pufferlösung vom pH = 8.6 und Ionenstärke $\mu = 0.012$ verwendet. Das Filtrierpapier war Munktell No. 20/150, die Zeit zwischen Eingang und Ausgang der Substanz war 3.5 Stunden (die vertikale Puffergeschwindigkeit), die Stromspannung 400 V, die Stromstärke 20 mA. Die Grösse des Filtrierpapiers kann aus Fig. 9 entnommen werden. In Fig. 36 ist jeweils auf der linken Seite ein Chromatogramm, und unten eine eindimensionale Elektrochromatographie derselben Farbstoffmischung abgebildet. Es ist unter anderem aus Fig. 36 ersichtlich, dass Fluorescein (F) eine grössere elektrophoretische Beweglichkeit besitzt als Chlorphenolrot (P), während die elektrochromatographische Beweglichkeit des Fluoresceins (F) kleiner als die des Chlorphenolrots (P) ist. Tropäolin O (T) unterscheidet sich hinsichtlich der elektrophoretischen Beweglichkeit viel mehr vom Chlorphenolrot (P) als bezüglich der elektrochromatographischen Beweglichkeit. Rosolsäure (R) wird elektrokinetisch in zwei Fraktionen (R_1 und R_2) getrennt. Die elektrophoretische Beweglichkeit der beiden Fraktionen ist bedeutend grösser als ihre elektrochromatographischen Beweglichkeiten.

STRAIN[12,73] bezweifelt die Behauptung, der chromatographische Effekt sei derselbe in der horizontalen wie auch in der vertikalen Richtung. Die chromatographische Retardation in der Richtung des elektrischen Stromes sollte sich von der chromatographischen Retardation in der Richtung senkrecht zum elektrischen Strom unterscheiden. Die Tatsache, das sich z.B. Ag und Ni Ionen in 4 M NH_4OH getrennt am Filtrierpapier auf derselben Bahn befinden[7] sei noch kein Beweis daführ, dass diese Ionen unter den gegebenen Bedingungen identische elektrophoretische Beweglichkeiten aufweisen, zumal sie sehr verschieden stark am Trennbogen adsorbiert werden[73]. Demzufolge sollte bei der kontinuierlichen Elektrophorese neben der elektrophoretischen Beweglichkeit die Adsorption am Träger doch noch eine bestimmte Rolle im Endergebnis der kontinuierlichen Trennung spielen. Dieses würde dann den Ausdruck "kontinuierliche Elektrochromatographie"[7,50,74,75] rechtfertigen. Neulich versuchte auch ZWEIG[76] den Ausdruck "kontinuierliche Elektrochromatographie" zu rechtfertigen, in dem er eine kontinuierliche Trennung von zwei verschiedentlich adsorbierbaren Farbstoffen, Brillantblau und Amarant, am Zellulosepapier und Glasfaserpapier angab. Brillantblau wanderte bei pH 8.6 in der Richtung der Kathode im Glasfaserpapier, während es im Zellulosepapier eine schwach anionische Beweglichkeit zeigte. Amarant wanderte dagegen in beiden Trägern anionisch. Dieses Beispiel braucht aber unbedingt keine Anomalie der Wanderung des Brillantblaus zu veranschaulichen, da im alkalischen Medium das Glasfaserpapier bekanntlich eine viel grössere Elektroosmose aufweist als das Zellulosepapier[77]. Beim Glasfaserpapier ist dann unter den angegebenen Bedingungen der gegengerichtete Horizontalvektor w' viel grösser als beim Zellulosepapier. Die bessere kontinuierliche Trennung der beiden Farbstoffe am Glasfaserpapier könnte vermutlich durch kleinere vertikale Puffergeschwindigkeit W im Glasfaserpapier zustande kommen, was unter gleichbleibenden Experimentalbedingungen zu relativ grösseren Neigungen der Bahnen gegen die Vertikale führen kann.

3. *Blockierung der Elektroden durch eine Membrane*

Wenn der Trennbogen durch eine Membrane, z.B. Cellophanstreifen oder dergl., von der Elektrodenflüssigkeit getrennt wird, werden nach PUČAR[78] die horizontale oder elektrochromatographische und auch vertikale oder chromatographische Komponente der Bahnen grundsätzlich verändert. Die Cellophanmembrane verhindert in grossem Ausmass die Kapillarsaugwirkung des Filtrierpapiers in horizontaler Richtung, und das Filtrierpapier kann den durch Stromwärme bzw. Verdunstung entstandenen Verlust des Wassers in der Horizontalrichtung durch Ansaugen der Elektrolytlösung aus den Elektrodenrinnen nicht ersetzen. Es können sich in der Horizontalrichtung keine Gradienten der Elektrolytkonzentration ausbilden, da die kapillare Elektrolytgeschwindigkeit in der Horizontalrichtung gleich Null wird ($w' = 0$), und demzufolge die elektrische Feldstärke (H) am Trennbogen in der Horizontalrichtung konstant ist ($dH/ds = 0$). Die vertikale Elektrolytgeschwindigkeit (W) ist, im Gegenteil zu der im vorangegangenen Kapitel bereits besprochenen Arbeitsweise, keinesfalls konstant, sie fällt in der Richtung von oben nach unten wegen Verdunstung und Konzentrierung des Elektrolyts ab.

Diese Verminderung der Vertikalgeschwindigkeit ist begreiflicherweise eine Funktion der Elektrolytleitfähigkeit und der elektrischen Feldstärke allein und sie ist unabhängig von der Filtrierpapierdicke. Die Neigungen der Bahnen der Teilchen gegen die Vertikale wachsen stetig in der Richtung von oben nach unten, da der Tangens des Neigungswinkels $\text{tg}\,\alpha = uH/W$ ist.

Je grösser die Elektrolytleitfähigkeit und die Feldstärke gewählt werden, desto schneller wachsen die Neigungen der Bahnen in der Richtung von oben nach unten (Fig. 37).

Fig. 38 gibt als Funktion der Zeit die Vertikalgeschwindigkeiten der Farbstofffronten des Chlorphenolrots wieder, die alle 15 Minuten gemessen wurden. Unter den angegebenen experimentellen Bedingungen hat Chlorphenolrot keinen chromatographischen Effekt am Filtrierpapier ($R_F = 1$).

Die Längen "a" und "b" sind die entsprechenden Durchlaufzeiten, d.h. die zwischen Einlauf und Auslauf der Substanz aus der Apparatur abgelaufene Zeit. Mit der Zeit, d.h. je mehr die Teilchen in der Vertikalrichtung nach unten fortschreiten, fällt die Vertikalgeschwindigkeit, d.h. die Vertikalkomponente, in dem Masse ab, wie es die verwendete elektrische Feldstärke und Elektrolytleitfähigkeit bestimmen, und die Neigungen der Bahnen gegen die Vertikale werden immer grösser. Deswegen sind die Krümmungen der Bahnen in Fig. 37 rechts grösser als in Fig. 37 links.

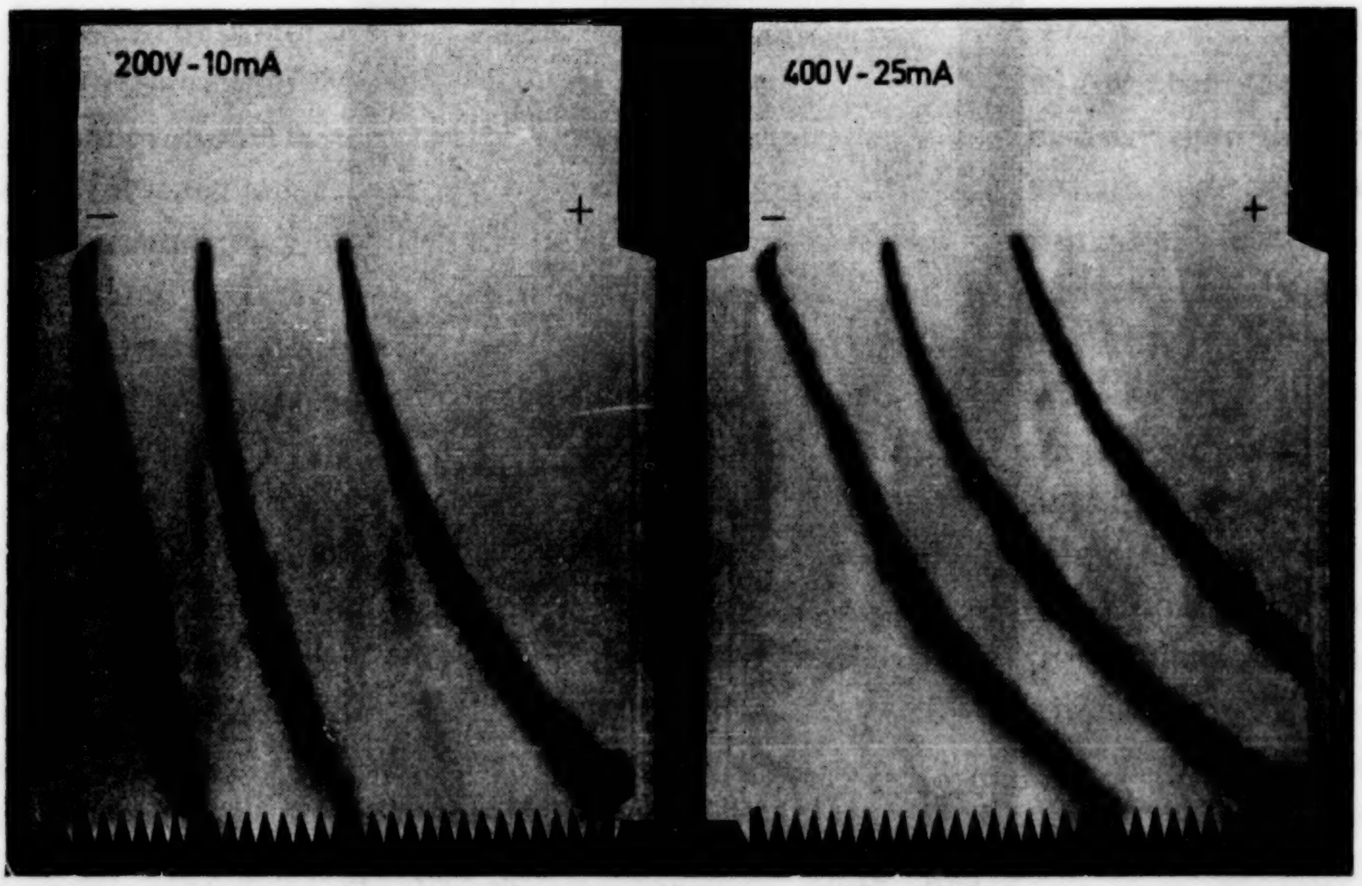


Fig. 37. Kontinuierliche Elektrophorese des Chlorphenolrots in Borsäure–Na-Acetat–NaOH mit Blockierung der Elektroden durch eine Cellophanmembrane nach PUČAR[78]. Pufferlösung von pH = 8.6 und Ionenstärke $\mu = 0.012$. Filtrierpapier Munktell No. 20/150. Die Farbstofflösung wurde gleichzeitig an drei verschiedenen Stellen der Apparatur zugeführt. Links: 200 V und 10 mA. Die Durchlaufzeit war 3 Std. 45 Min. Rechts: 400 V und 25 mA. Die Durchlaufzeit war 4 Std. 15 Min. Die Krümmungen der Bahnen sind wegen grösserer elektrischer Feldstärke im rechten Bild grösser als im linken Bild. Die Bahnen laufen annähernd parallel. Chlorphenolrot wird elektrophoretisch in zwei Fraktionen zerlegt: in eine dunkelrote mit grösserer Beweglichkeit (im Bilde gut sichtbar), und eine gelbe mit viel kleinerer Beweglichkeit.

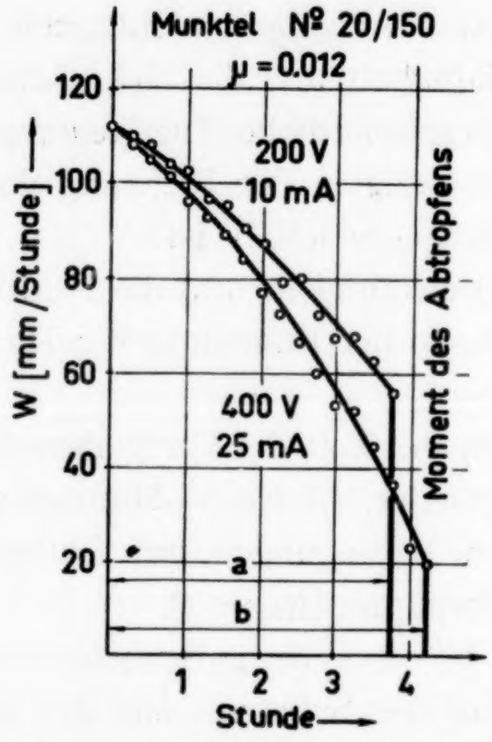


Fig. 38. Die vertikale Komponente (W) nach PUČAR[78] als Funktion der Zeit und auf ein der Elektrophorese unterworfenes Teilchen bezogen. In Experimenten, die in Fig. 37 wiedergegeben sind, wurden die Vertikalgeschwindigkeiten der Farbstoffronten alle 15 Minuten gemessen. "a" und "b" sind die Durchlaufzeiten für das in Fig. 37 links, bzw. rechts, wiedergegebene Experiment.

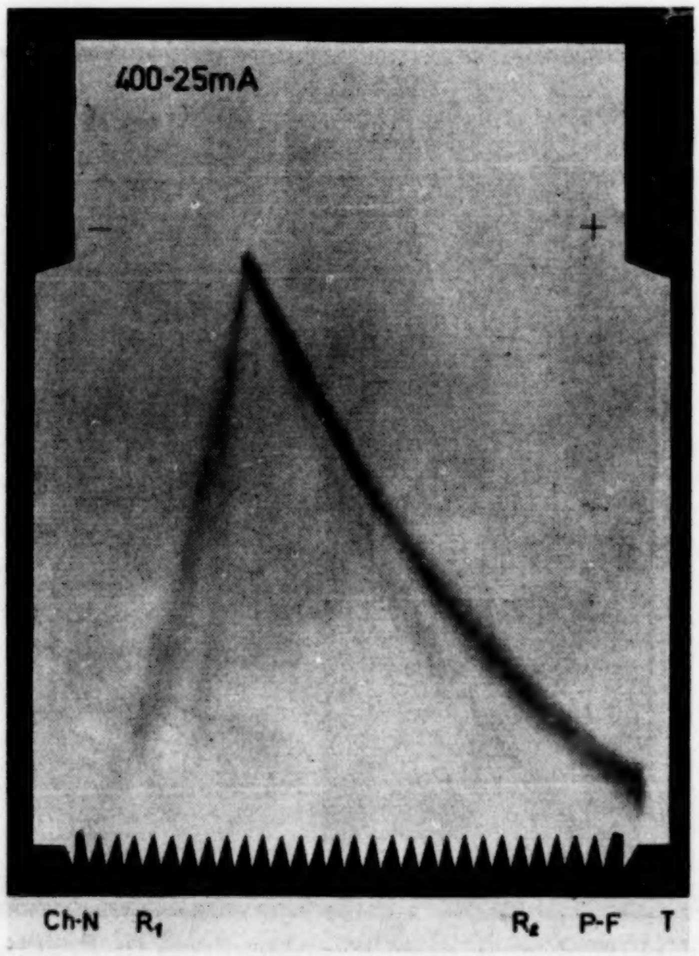


Fig. 39. Kontinuierliche Elektrophorese einer Mischung von Tropäolin O(T), Fluorescein (F), Chlorphenolrot (P), Rosolsäure (R_1 und R_2), Chrysoidin Y(Ch) und Nitranilin (N) mit Membranblockierung nach PUČAR[78]. Alle Experimentaldaten wie im Experiment Fig. 37 rechts. Dieses Experiment entspricht dem in Fig. 36 links wiedergegebenen. Da auch die Substanzzufuhrstelle die gleiche war, sind die beiden Abbildungen unmittelbar miteinander vergleichbar. Man beachte die Durchlaufzeiten und Stromstärken: Ohne Membran 3 Std. 30 Min. und 20 mA (Fig. 36), und mit Membranblockierung (Fig. 38) 4 Std. 15 Min. und 25 mA. Wegen grösserer Krümmungen der Bahnen sind alle Fraktionen bedeutend weiter gewandert.

In Fig. 39 wurde das Experiment aus Fig. 36 links wiederholt, aber jetzt mit Membrantechnik. Durchlaufzeit und Stromstärke sind bedeutend gewachsen: ohne Membran 3 Stunden 30 Minuten und 20 mA und mit Membranblockierung 4 Stunden 15 Minuten und 25 mA. Da auch die Substanzzufuhrstelle dieselbe war, sind die beiden Abbildungen unmittelbar vergleichbar. Wegen grösserer Krümmungen der Bahnen sind alle Fraktionen jetzt weiter gewandert.

Charakteristisch für die kontinuierliche Elektrophorese in feuchter Kammer mit Membranblockierung der Elektroden ist, dass je nach dem Arbeitsregime alle Bahnen ausnahmslos kleinere oder grössere Krümmungen im Sinne der apparenten Beschleunigung aufweisen. Sie hat bei kontinuierlichen Arbeiten einen grossen praktischen Vorteil, da das Dichten der Elektrodenrinnen durch Aufkleben der Membranen vollkommen ist, und demzufolge kein Verlust der Pufferlösung auftritt, was von ausschlaggebender Bedeutung für das aufsichtslose kontinuierliche Spülen der Elektrodenrinnen im Dauerbetrieb ist.

(*b*) *Terminologie*

In der Literatur wurden bereits mehrere Ausdrücke für die verschiedenen elektrophoretischen und elektrochromatographischen Prozesse am Filtrierpapier und anderen Trägersubstanzen vorgeschlagen, wie z.B. Ionographie[79], Papierelektrophorese[36,71,72,80-83], Papierionophorese[81], kontinuierliche Elektrophorese[6,36,78,84,120-132], kontinuierliche Ionophorese[6], Elektrochromatographie[7,36,50,74], kontinuierliche Elektrochromatographie[7,21,50,74], Zonenelektrophorese[10], Elektrochromatophorese[36,85], Elektromigration[86] u.s.w. Entsprechend einem Vorschlag von PUČAR[36] sind die Ausdrücke für einzelne Prozesse in Tabelle I wiedergegeben. Falls man sich bezüglich der Auffassung, dass die adsorptive Retardationen in der Richtung des elektrischen Stromes und senkrecht zu dieser Richtung miteinander identisch sind, allgemein einigen könnte, müsste man, laut den Ausführungen im Abschnitt über die vertikale oder chromatographische Komponente, den Ausdruck "kontinuierliche Elektrochromatographie" vollkommen aufgeben, da die chromatographische Komponente im kontinuierlichen Prozess dann keinen Einfluss auf die Trennung hat. Die eindimensionale Elektrophorese, bzw. Streifenelektrophorese in porösen Trägern ist ein Prozess, bei welchem die chromatographischen Effekte nicht zum Ausdruck kommen (sämtliche Fraktionen haben $R_F = 1$, oder $R_F \sim 1$), wie z.B. bei der üblichen Papierelektrophorese der Serumeiweisse und Kohlenhydrate[87-89]. Die übliche eindimensionale Trennung der Serumlipoide ist dagegen ein elektrochromatographischer Prozess[88,89]. Wenn die Fraktionen einen merklichen chromatographischen Effekt zeigen ($R_F = 1$), wäre der Ausdruck "Streifenelektrophorese" nur dann zulässig, falls die Resultate, z.B. auf rechnerischem Wege, frei von chromatographischen Effekten angegeben werden, wie z.B. bei der Ermittlung elektrophoretischer Beweglichkeiten[90-93].

Der Ausdruck "Ionophorese", der den Unterschied zwischen Ionenwanderung und Wanderung kolloider Partikelchen unterstreichen sollte, müsste nach dem Vorschlag von PUČAR[36] umgangen werden, weil: (1) elektrokinetisch im wesentlichen kein Unterschied besteht, (2) häufig ist es unmöglich, eine scharfe Grenze zwischen Kolloidteilchen und Ionen aufzustellen, besonders bei biologischem Material, (3) man kann die Wanderung der Kolloidteilchen nicht als Ionophorese bezeichnen, im Gegenteil, es ist durchaus zulässig die Wanderung der Ionen als Elektrophorese zu betrachten.

Es muss darauf hingewiesen werden, dass der Ausdruck "zweidimensionale Elektrochromatophorese" oder "zweidimensionale Elektrochromatographie" nicht mit der Elektrochromatographie in zwei Richtungen zu verwechseln ist, da die Elektrochromatographie in zwei (oder mehreren) Richtungen, wie sie DURRUM[94] oder STRAIN[95] beschrieben haben, nicht gleichzeitig (simultan) in beiden Richtungen verlaufen kann. Diese Technik ist lediglich aus zwei eindimensionalen elektrochromatographischen Prozessen zusammengestellt. Dasselbe gilt auch für die Kombination von Papierelektrophorese und Papierchromatographie wie sie KICKHÖFEN UND WESTPHAL[96] beschrieben haben. In diesem Falle ist die Technik aus einem elektrophoretischen oder elektrochromatographischen und einem separaten chromatographischen Prozess zusammengestellt.

4. EINIGE EXPERIMENTELLE ANWENDUNGSBEISPIELE

(*a*) *Kontinuierliche elektrophoretische Trennungen*

1. *Farbstoffgemische*

Für die ersten kontinuierlichen elektrophoretischen Trennungen wurden Farbstoffgemische verwendet. Durch Farbstofftrennungen haben die meisten Autoren auf einfachste Weise das Prinzip des Verfahrens und gleichzeitig das Funktionieren ihrer eigenen Trennapparate untersucht. Im Acetatpuffer von pH 5.3 haben SVENSSON UND BRATTSTEN[4] und GRASSMANN[51] die Brauchbarkeit ihrer ersten Apparate mit küvettenförmiger Trennkammer an Hand der Farbstofftrennung Bromphenolblau–Bromthymolblau erwiesen. Am Filtrierpapier hat als erster GRASSMANN[51] eine Trennung von Kresolrot–Bromphenolblau in $M/15$ Phosphatpuffer bei pH 6.9 angegeben. DURRUM[6] trennte eine Lösung von saurem Fuchsin in $N/4$ Essigsäure in 7 sichtbare und 3 weitere, erst im Ultraviolett sichtbare Komponenten. In der gleichen Grundelektrolytlösung trennte DURRUM[6] auch Acridinorange von einer nichtfluoreszierenden roten Unreinheit, obwohl die beiden Komponenten am Filtrierpapier sehr stark adsorbierbar waren. In $4\,M$ Ammoniak trennten STRAIN UND SULLIVAN[7] Methylorange von Phenolphthalein. GRASSMANN UND HÜBNER[119] trennten einige kommerzielle saure und basische Farbstoffe der Firma BASF, Bayer-Leverkusen, in Komponenten. Im Boratpuffer vom pH 8.6 trennte PUČAR[36, 78] eine Mischung von Tropäolin O, Fluorescein, Chlorphenolrot, Rosolsäure, Chrysoidin Y und Nitranilin (vergl. Fig. 36 und 39). GRASSMANN UND HANNIG beschrieben die Auftrennung einer synthetischen Mischung von Bromphenolblau, Bromkresolgrün, Kresolrot, Fuchsin S und Kresolpurpur im Essigsäure–Pyridin-puffer bei pH 4.9[20, 33, 38], und die Auftrennung einer Mono-, Di, Tri- und Tetrasulfonsäure eines Phthalocyanins in 1 N Essigsäure[20]. In Acetatpuffer bei pH 5.3 trennten DOBRY UND FINN[57] eine Mischung aus basischem Fuchsin, Bromthymolblau und Bromphenolblau. BARROLLIER, WATZKE UND GIBIAN[60] trennten in 0.04 *M* Ammonacetat bei pH 5.6 die Mischung Neucoccin–Bromphenolblau–Uranin. Brillantblau–Amarant wurde in Veronalpuffer bei pH 8.6 und Ionenstärke 0.05 von ZWEIG[76] aufgetrennt.

2. *Einfache und zusammengesetzte Eiweisstoffe*

Die kontinuierliche Trennung der gefärbten Proteine Phycoerythrin–Phycocyanin stellt, neben den etwas später erfolgten Trennungen der farblosen Serumproteine, eine klassische präparative Proteintrennung durch kontinuierliche Elektrophorese dar. In Phosphatpuffer vom pH 7.7 und Ionenstärke 0.025 und 0.050 trennten SVENSSON UND BRATTSTEN[4] eine künstliche Mischung von Phycoerythrin und Phycocyanin erfolgreich in Komponenten, und auch die natürlichen Extrakte der Alge, in der diese Proteine vorkommen. Die ersten Trennungen der Serumproteine in Veronalnatrium, Veronalnatrium–Natriumacetat oder Phosphatpuffer bei pH 8.6 bzw. pH 7.7, wurden von DURRUM[6], GRASSMANN UND HANNIG[5, 34, 51] und BRATTSTEN UND NILSSON[28] angegeben. In einer Reihe weiterer Arbeiten trennte BRATTSTEN in seinem Pumpenapparat unter verschiedenen experimentellen Bedingungen grössere Mengen von Serumeiweiss[53, 54, 100] (vergleiche dazu auch Fig. 26), die Algenproteine Phycoerythrin und Phycocyanin[54], und Proteine des cerebrospinalen Liquors[54].

Die Toxine zahlreicher Giftschlangen wurden im Veronal–Natriumacetat-Puffer bei pH 8.6 und Ionenstärke 0.05 von GRASSMANN UND HANNIG[97] kontinuierlich getrennt. Alle untersuchten Toxine erwiesen sich als Gemische einer Anzahl elektrophoretisch auftrennbarer Einzelkomponenten, denen zum Teil eine bestimmte Enzymwirkung, wie z.B. Protease, Lecithinase u.s.w., zugeordnet werden konnte. Ein Cyanokobalamin–Protein-Komplex wurde nach Zugabe des Vitamins zur Milch des Schweines, der Ratte, der Ziege, der Kuh und zu Frauenmilch durch kontinuierliche Elektrophorese im 0.025 *M* Na-Veronalpuffer bei pH 8.6 von GREGORY UND HOLDSWORTH[107] rein erhalten.

OSTROWSKI UND NIEWIAROWSKA-PAWLUS[30] und SKARZYNSKI UND OSTROWSKI[108] reinigten einen im Rindserum vorkommenden Vitamin B_{12}–Protein-Komplex (Erythroglobulin) bei pH 8.6, und erhielten eine 625-fache Anreicherung des Vitaminkomplexes.

Eine künstliche Mischung von zwei Stämmen des Tabakmosaikvirus U_1 und U_2 trennte ZAITLIN[109] kontinuierlich in *M*/90 Phosphatpuffer bei pH 6.9. Eine Reinigung desselben Virus unter gleichen experimentellen Bedingungen wurde auch angestrebt[110]. Die Reinigung der infektiven Nukleinsäure aus dem Tabakmosaikvirus im Phosphatpuffer bei pH 7.3 wurde von TAKAHASHI, KARLER UND KNIGHT[111] beschrieben.

BAMANN UND TIETZ[112] haben eine kontinuierliche Trennung der Samenlipase von *Nigella sativa* in Veronal–Natriumacetat-Puffer bei pH 8.4 angegeben. Im γ-Globulinbereich wurden zwei Globulinfraktionen erhalten. PFEIL UND KANNGIESSER[113] haben aus einem Eiweisspräparat aus Kartoffelknollen im Michaelispuffer bei pH 8.6 und nach Anfärbung mit Ninhydrin fünf sichtbare Fraktionen erhalten. Proteine von verschiedenen Karzinomen wurden von DOMBROWSKI[114] in Veronal–Na-Veronal-Puffer bei pH 8.6 getrennt und durch Dialysieren und Gefriertrocknung rein dargestellt.

KARLER UND SPIVACK[115, 116] verwendeten sehr wirkungsvoll die kontinuierliche Elektrophorese bei Reinigung grosser Mengen des rohen Toxins der *Pasteurella pestis*. Auch bei mehrmahliger Fraktionierung konnte man keinen Verlust des Toxins feststellen. KARLER[117] hat auch eine Reinigung des Tetanus-Toxoids beschrieben, sowie eine Fraktionierung des vollen Homogenates von *Pseudomonas fluorescens*. Im letzteren Falle wurden 6 verschiedene Enzyme erhalten und identifiziert.

Eine kontinuierliche elektrophoretische Reinigung eines Trypsinpräparates im Pyridinpuffer bei pH 6.1 wurde von GRASSMANN, HANNIG UND SCHLEYER[118] beschrieben. Die vollständige Abtrennung des stets das Trypsin begleitenden Chymotrypsins konnte erreicht werden.

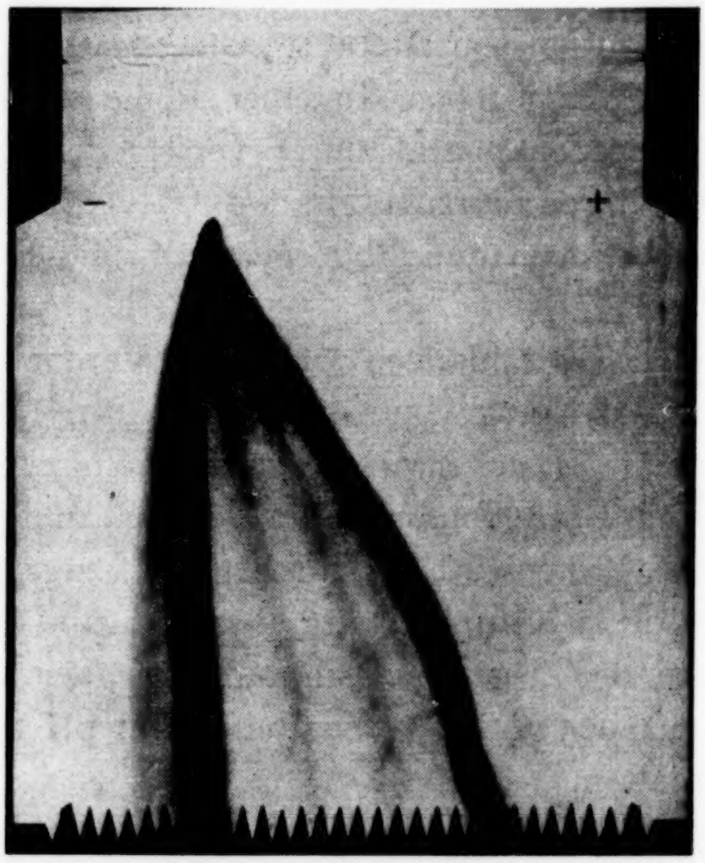


Fig. 40. Trennung eines pathologischen menschlichen Serums (γ-Plasmocytom) in Veronalpuffer bei pH 8.6 nach PUČAR[36].

Ausser den bereits erwähnten Autoren haben auch zahlreiche andere Forscher eine kontinuierliche Trennung der Serumproteine durchgeführt und beschrieben[20, 31, 33, 36, 38, 60, 62, 120–132, 143, 147]. Die getrennten Proteinfraktionen wurden entweder dialysiert und einer Gefriertrocknung unterworfen, oder sie wurden immunochemisch oder auch analytisch weiter differenziert und identifiziert, bzw. auf enzymatische Wirkung innerhalb der einzelnen Fraktionen geprüft.

3. *Aminosäuren und Peptiden*

GRASSMANN UND HANNIG[5, 51] haben zahlreiche kontinuierliche Trennungen von verschiedenen Aminosäuregemischen bereits in den ersten Jahren der kontinuierlichen Elektrophorese angegeben. Getrennt wurden folgende Gemische: Glutaminsäure–Asparaginsäure in $M/100$ Zitratpuffer bei pH 3.5 und $M/10$ Zitratpuffer bei pH 3.79, Glutaminsäure–Alanin–Histidin in $M/15$ Phosphatpuffer bei pH 5.2, Lysin–Histidin bei pH 6.8, Leucin–Glutaminsäure bei pH 5.2, Glykokoll–Alanin bei pH 11.5, Alanin–Threonin bei pH 11.0, Alanin–Cystin bei pH 11.5, Arginin–Histidin–Alanin–Asparaginsäure bei pH 7.4, Lysin–Alanin–Threonin–Glutaminsäure bei pH 8.5 u.s.w. Die teilweise unvollkommene Trennung einer Mischung von 9 Aminosäuren Arginin–Glycin–Alanin–Valin–Serin–Threonin–Phenylalanin–Prolin–Asparagin in N Essigsäure wurde in den ersten Jahren (1951) von DURRUM[6] beschrieben. Im selben Jahre trennten BRATTSTEN UND NILSSON[28] in Ameisensäurepuffer bei pH 2.61 eine Mischung von Glycin, Alanin und Glycyl-glycin. GRASSMANN und Mitarb.[34, 98] befassten sich anschliessend an die Trennung der Aminosäuren mit der Trennung von Hydrolysaten von Casein, Kollagen, Prokollagen und Insulin zwecks Identifizierung und auch quantitativer Bestimmung der Aminosäuren. Die Aminosäuren der Hydrolysate werden zweckdienlich im ersten Gange einer kontinuierlichen Gruppentrennung unterworfen (Fig. 41). Diese gelingt am besten bei pH 3.9 in Gegenwart von Acetatpuffer. Unter diesen Bedingungen wandern Cysteinsäure, Asparaginsäure und Glutaminsäure vollkommen voneinander getrennt anodisch, während die sämtlichen neutralen Aminosäuren ungetrennt in einer relativ schmalen, ganz schwach kathodisch wandernden Mittelzone verbleiben. Die basischen Aminosäuren Histidin, Lysin und Arginin wandern scharf von den anderen getrennt nach der Kathodenseite. Die Trennung der neutralen Aminosäuren wird am besten in 1 N Essigsäure bei pH 2.4 vorgenommen. In Fig. 42 ist die Trennung von 10 neutralen Aminosäuren wiedergegeben: Glykokoll, Alanin, Valin, Leucin, Serin, Threonin, Methionin, Phenylalanin, Prolin und Oxyprolin. Die Trennung der basischen Aminosäuren Histidin,

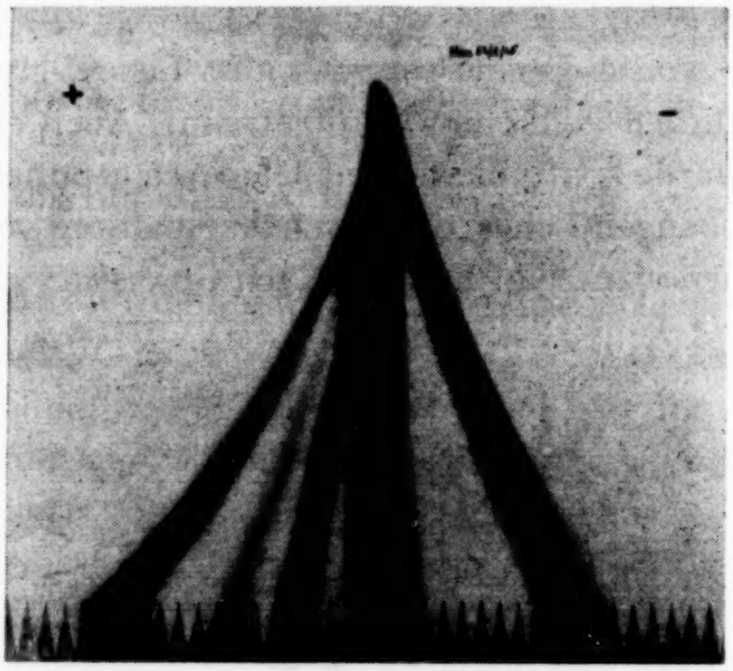
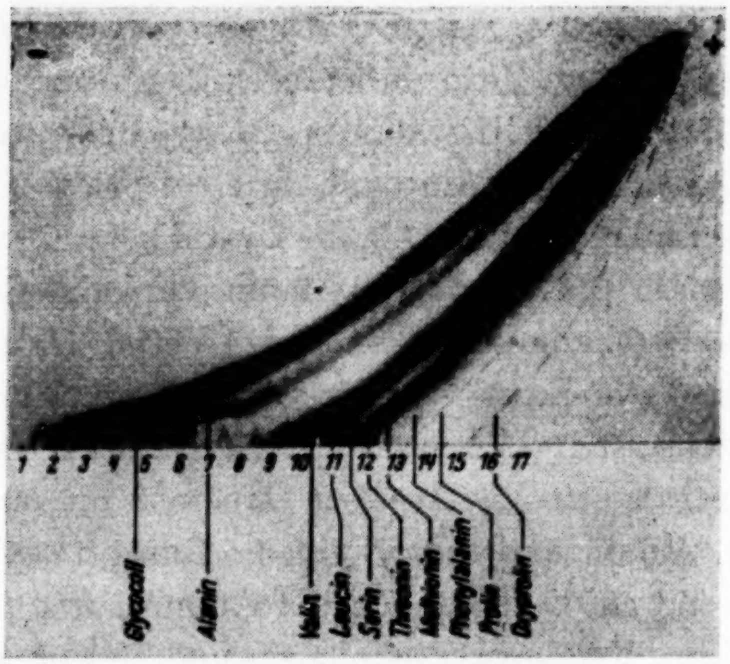


Fig. 41. Gruppentrennung eines Eiweisshydrolysates nach GRASSMANN, HANNIG UND PLÖCKL[98]. Fraktion B nach Perameisensäure-Oxydation von Insulin. Pyridin–Essigsäure-Puffer pH 3.9, Papier Schleicher & Schüll 2040a, 290 V, 10 mA. Von links nach rechts: Cysteinsäure, Asparaginsäure, Glutaminsäure, neutrale Aminosäuren, basische Aminosäuren.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16
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Alanin
Valin
Leucin
Serin
Threonin
Methionin
Phenylalanin
Prolin
Oxyprolin

Fig. 42. Trennung der neutralen Aminosäuren in 1*N* Essigsäure bei pH 2.4 nach GRASSMANN UND HANNIG[34]. Trennfläche 48 × 28 cm, Schleicher & Schüll 2040b, 700 V, 13 mA.

Lysin und Arginin wird in *M*/25 Boratpuffer bei pH 10.5 vorgenommen (Fig. 43).

Trennungen von Peptiden werden nach GRASSMANN UND HANNIG[34] in 1 *N* Essigsäure bei pH 2.4 ausgeführt. Mischungen von Glykokoll, Glycyl-glycin und Diglycyl-glycin, sowie von L-Leucyl-L-tyrosin, DL-Leucyl-glycyl-glycin, Glykokoll und Diglycyl-glycin wurden vollkommen aufgetrennt. Auch ein Abbaugemisch von Kollagen mit Trypsin wurde kontinuierlich im *M*/20 Acetatpuffer bei pH 5.0 getrennt[34]. Die Trennung eines Abbaugemisches, das durch Aufspaltung von Prokollagen mittels krist. Trypsins gewonnen war, zeigt Fig. 44. Nach GRASSMANN und Mitarb.[99] handelt

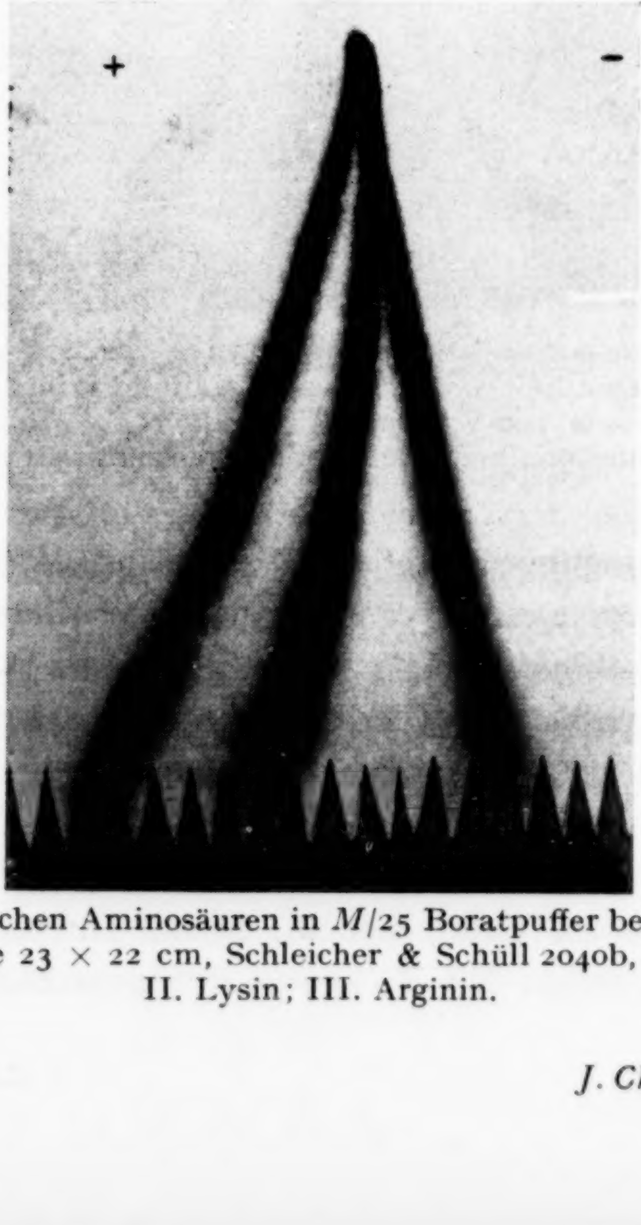

Fig. 43. Trennung der basischen Aminosäuren in *M*/25 Boratpuffer bei pH 10.5 nach GRASSMANN UND HANNIG[34]. Trennfläche 23 × 22 cm, Schleicher & Schüll 2040b, 140 V, 10 mA. I. Histidin; II. Lysin; III. Arginin.

es sich hier um Peptide mit einer mittleren Kettenlänge von etwa 18 Aminosäuren. Die Trennungen wurden bei pH 2.3, 4.9 und 9.2 am Papier Schleicher & Schüll 604 L und unter Dosierung von 250 mg des Abbaugemisches in 24 Stunden durchgeführt. GRASSMANN und Mitarb.[106] haben auch die durch Perameisensäure-Abbau erhaltenen Fraktionen A und B des oxydierten Insulins in 1 *N* Ameisensäure quantitativ getrennt.

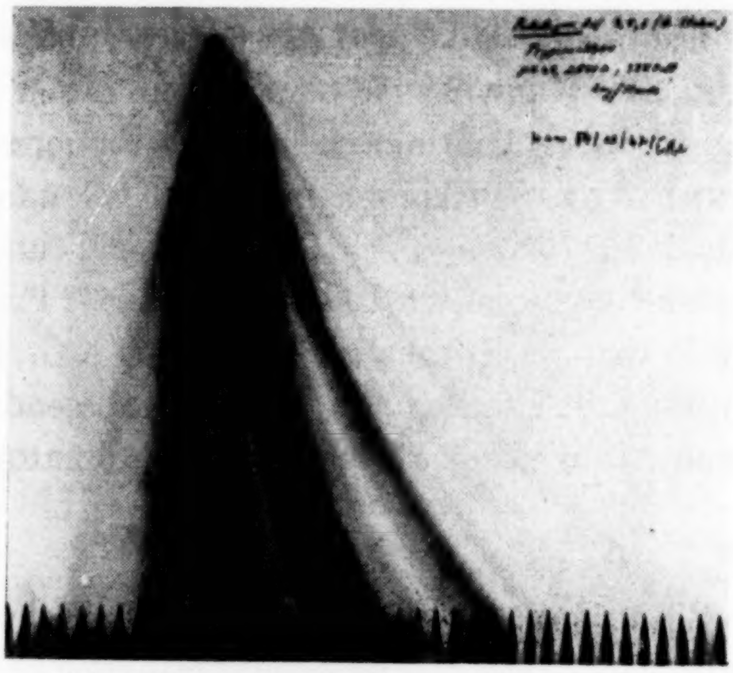

Fig. 44. Auftrennung eines mit krist. Trypsin erhaltenen Abbaugemisches von Prokollagen (Peptide) in Pyridin–Essigsäure-Puffer bei pH 4.9 nach GRASSMANN u. Mitarb.[20,99]. 375 V, 20 mA.

STEGEMANN[105] trennte Proteinhydrolysate mit basischen, neutralen und sauren Aminosäuren an einem PVC-Papier (Schleicher & Schüll; Rhovylpapier No. 1001) im 0.02 *M* Phosphatpuffer bei pH 7.1. Für die Pufferlösung wird, wegen besserer Benetzung des PVC-Materials, eine Mischung von Wasser und Äthylenglykolmonomethyläther (Methylcellosolve) im Verhältnis 3:7 verwendet.

Die kontinuierliche Elektrophorese wurde für präparative Gewinnung der durch Hydrolyse mittels Trypsin gewonnenen Peptide aus kristallisiertem humanen Hämoglobin A verwendet. LIEBOLD und Mitarb.[133] benutzten als Grundelektrolyt 0.5 *N* Essigsäure (pH 2.5–2.6).

4. *Sonstige Trennungen organischer Stoffe*

MICHEEL UND VAN DE KAMP[134] trennten Zuckergemische in Form von Borsäure-Komplexen in *M*/30 Borsäure, die mit NaOH auf pH 9.2 gebracht wurde. Es wurde eine Mischung von Cellobiose, L-Rhamnose, D-Mannose und D-Glukose, und ausserdem ein aus D-Fructose und D-Glukose bestehendes Inulin-Hydrolysat kontinuierlich getrennt. MOULD UND SYNGE[135] trennten die Hydrolysenprodukte der durch α-Amylase hydrolysierten Amylose, und zwar in Form von Jodkomplexen. In einem 0.04 *N* Acetatpuffer von pH 4.8 mit 0.001 *N* J_2 und 0.002 *N* KJ wurden 3 Fraktionen erhalten, eine blaue, rote und orangegelbe. GRASSMANN UND HANNIG[34] und GRASSMANN UND STADLER[136] trennten kontinuierlich Gemische natürlicher und synthetischer Gerbstoffe in *M*/30 Acetatpuffer bei pH 5.0.

Die Trennung von 100 mg einer Mischung der Alkaloide Scopolamin und Hyoscyamin wurde in Veronalpuffer bei pH 8.6 in 40 Stunden von DECKERS UND SCHREIBER[137] durchgeführt. WEISGLASS, PUČAR UND PETEK[138] trennten kontinuierlich im 0.012 *N* Ammonacetat Äther- und Chloroformextrakte von Nährböden, in denen *Pseudomonas pyocyanea* wuchs. Dabei zeigten sich 3 farbige Fraktionen, 8 im U.V.-Licht fluoreszierende und 7 im normalen und U.V.-Licht farblose, aber antibiotisch wirksame Fraktionen. OSTROWSKI UND KRAWCZYK[139] reinigten ein durch Extraktion gewonnenes B_{12}-Vitaminpräparat mittels kontinuierlicher Elektrophorese in 0.5 *N* Essigsäure. LEDERER UND MARINI-BETTOLO[16] trennten in 25% Essigsäure die im U.V.-Licht fluoreszierenden Alkaloide aus *Strychnos froesii*. Im Veronal- und Phosphatpuffer bei pH 8–8.6 und Ionenstärke 0.05 trennten UEDA UND MORI[146] Präparate des thyrotropischen Hormons. Das Präparat Pretiron der Fa. Schering A.G., Berlin, (18 mg) wurde in 3 Komponenten kontinuierlich aufgetrennt, und anschliessend wurden die HCl-Hydrolysate der Komponenten auf einzelne Zucker papierchromatographisch untersucht.

5. *Anorganische Ionen*

STRAIN UND SULLIVAN[7] waren die ersten, die über kontinuierliche Trennungen anorganischer Ionen berichteten (1951). Folgende, sehr erfolgreiche Trennungen, wurden angegeben: Ag–Cu in 0.1 *M* Milchsäure oder in einer Mischung von 4 *M* Ammoniak und 0.01 *M* EDTA, Ni–Cu und Ni–CrO_4^{--} in einer Mischung von 0.01 *M* Dinatriumtartrat und 4 *M* Ammoniak, Fe–Al in einer Mischung von 0.005 *M* Dimethylglyoxim, 0.01 *M* Weinsäure und 4 *M* Ammoniak, Sn–As–Sb in einer Mischung von 0.02 *M* Milchsäure, 0.02 *M* Weinsäure und 0.04 *M* DL-Alanin, Ag–Ni–Cu–Fe in einer Mischung von 0.01 *M* Dinatriumtartrat, 0.01 *M* Ammoniumoxalat und 4 *M* Ammoniak, Ag–Ni in einer Mischung von 0.01 *M* Ammoniumoxalat und 4 *N* Ammoniak oder noch besser in 0.005 *M* EDTA in 4 *M* Ammoniak. In einer späteren Mitteilung berichtete STRAIN[49] über weitere kontinuierliche Trennungen wie z.B.: Co–Fe und Mg–Fe in einer Mischung von 0.05 *M* Malonsäure und 0.1 *M* Essigsäure, Tl–Al in einer Mischung von 0.05 *M* Zitronensäure und 0.3 *M* Ammoniak, und Tl–As–Al und Ag–Cd–Bi–Al in einer Mischung von 0.05 *M* Malonsäure und 0.05 *M* Ammoniak.

SANSONI UND KLEMENT[101] studierten sehr eingehend Trennungen von kondensierten Phosphaten in einem NaOH–Na-Boratpuffer bei pH 10.0. Eine grössere Anzahl von sehr guten quantitativen Zweifachtrennungen und Mehrfachtrennungen der kondensierten Phosphate wurde angegeben. LEDERER[84] trennte Y–La und Pr–Nd in 1 % Zitronensäure. BIGHI UND TRABANELLI[102] trennten den α,α'-Dipyridyl-Komplex des zweiwertigen Eisens (Fe-Dip.) von Fe^{++} und Fe^{+++} oder von Ni oder Cr. Die Trennungen Fe^{+++}–(Fe-Dip.)–Fe^{++}, Ni–(Fe-Dip.), Fe^{+++}–Ni–(Fe-Dip) und Cr–(Fe-Dip.) wurden in 1% Zitronensäure oder 0.5%, bzw. 1%, K-Na-Tartrat durchgeführt. Ferner wurden auch Trennungen von Polythioanionen in 1% K-Na-Tartrat, wie z.B. Trithionat–Tetrathionat, und Trithionat–Pentathionat angegeben[102].

6. *Radioaktive anorganische Ionen*

Die kontinuierliche Trennung radioaktiver anorganischer Ionen wurde erstmals von

SATO, NORRIS UND STRAIN[50] angegeben. Folgende Mischungen wurden getrennt: ^{45}Ca–$^{32}PO_4$ in 0.1 *M* Milchsäure oder in 0.1 *M* Essigsäure[50], ^{90}Sr–^{90}Y in 0.1 *M* Milchsäure[103], ^{90}Sr–^{90}Y–$^{32}PO_4$, ^{45}Ca–^{90}Y–$^{32}PO_4$, ^{90}Sr–^{90}Y–^{144}Ce–$^{32}PO_4$, ^{137}Cs–^{90}Sr–^{90}Y–^{60}Co–$^{32}PO_4$ und ^{137}Cs–^{90}Sr–^{90}Y–^{233}U–^{95}Zr–^{95}Nb in 0.1 *M* Milchsäure[74]. LEDERER[84] trennte Ac von inaktivem La. PUČAR UND JAKOVAC[104] trennten zwei Mischungen von seltenen Erden, ^{144}Ce–^{160}Tb–^{170}Tm und ^{144}Ce–^{152}Eu–^{169}Yb, in 0.05 *M* Milchsäure (Fig. 45).

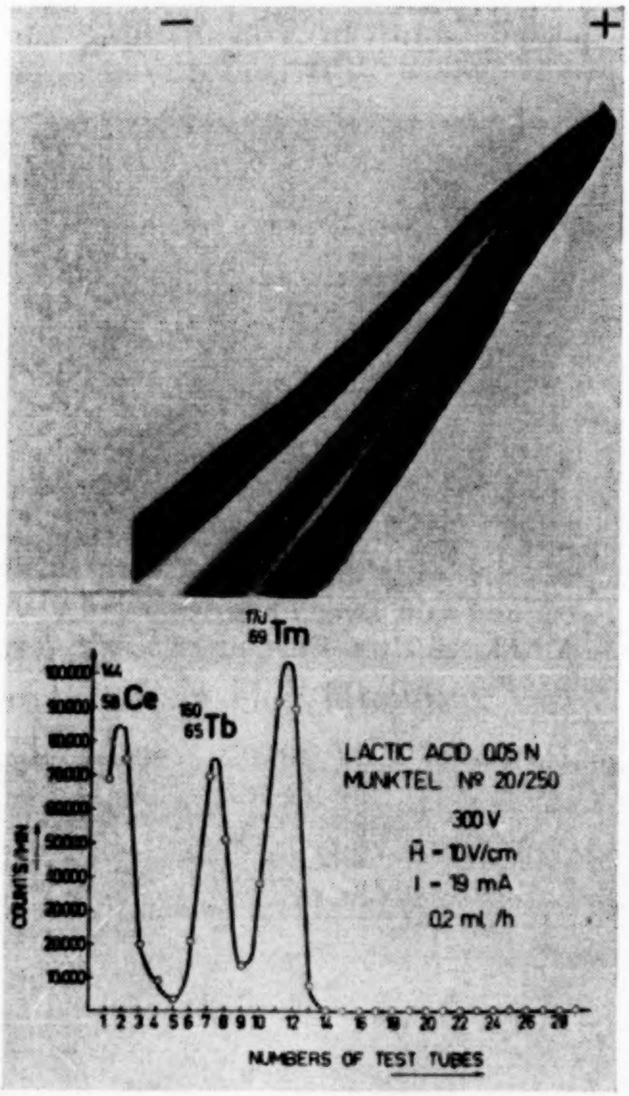

Fig. 45. Autoradiogramm einer kontinuierlichen elektrophoretischen Trennung von ^{144}Ce–^{160}Tb–^{170}Tm in 0.05 *M* Milchsäure nach PUČAR UND JAKOVAC[104]. Das unten aufgezeichnete Diagramm gibt die Aktivitäten in den Auffanggläsern wieder, die in einer Zeitperiode von 0.5 Stunden nach Einstellung des stationären Zustandes gesammelt wurden. Papier Munktell No. 20/250.

7. *Besondere Anwendungen*

Ob Additionsverbindungen oder lockere Komplexe zwischen zwei Stoffen gebildet werden oder nicht, kann man nach GRASSMANN UND HÜBNER[119, 141] unter Verwendung der kontinuierlichen Elektrophorese in einfacher Weise zeigen, wenn man beide Komponenten nicht, wie dies bei der normalen Trennung üblich ist, an einer Stelle in das Trennungsfeld einströmen lässt, sondern an zwei verschiedenen Stellen, und zwar derart, dass sich die Wanderungswege der beiden entgegengesetzt geladenen Stoffe kreuzen. Wenn keine Additionsverbindung gebildet wird, wandern die beiden Stoffe in geradlinigen Bahnen ungehindert durcheinander hindurch, wie z.B. das Farbstoffpaar Amidoschwarz–Fuchsin (Fig. 46 links); kommt es indessen zur Bildung einer Additionsverbindung, wie z.B. beim Farbstoffpaar Orange II–Methylenblau

(Fig. 46 rechts), dann werden die beiden Komponenten am Punkt der Kreuzung aus ihrer Wanderungsrichtung abgelenkt, und der entstehende Komplex bewegt sich nun, wenn er nicht unlöslich ausfällt, in einer mittleren Wanderungsrichtung. Im weiteren Verlauf trennen sich die beiden Komponenten wieder, und wandern in der ursprünglichen Richtung weiter, jedoch entsprechend ihrer Dissoziation aus dem Komplex in Form mehr oder weniger verwaschener und unscharfer Bahnen. Diese Methode modifizierten Nakamura und Ueta[142], indem sie die beiden Lösungen nicht kontinuierlich zuströmen liessen, sondern von Anfang an die beiden Lösungen in

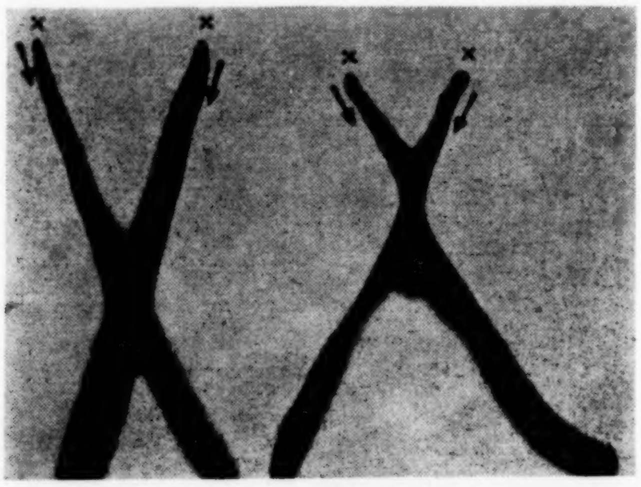

Fig. 46. Die kontinuierliche Kreuzung von zwei Farbstoffen in 1 N Essigsäure nach Grassmann und Hübner[141]. Links: Amidoschwarz–Fuchsin. Rechts: Orange II–Methylenblau.

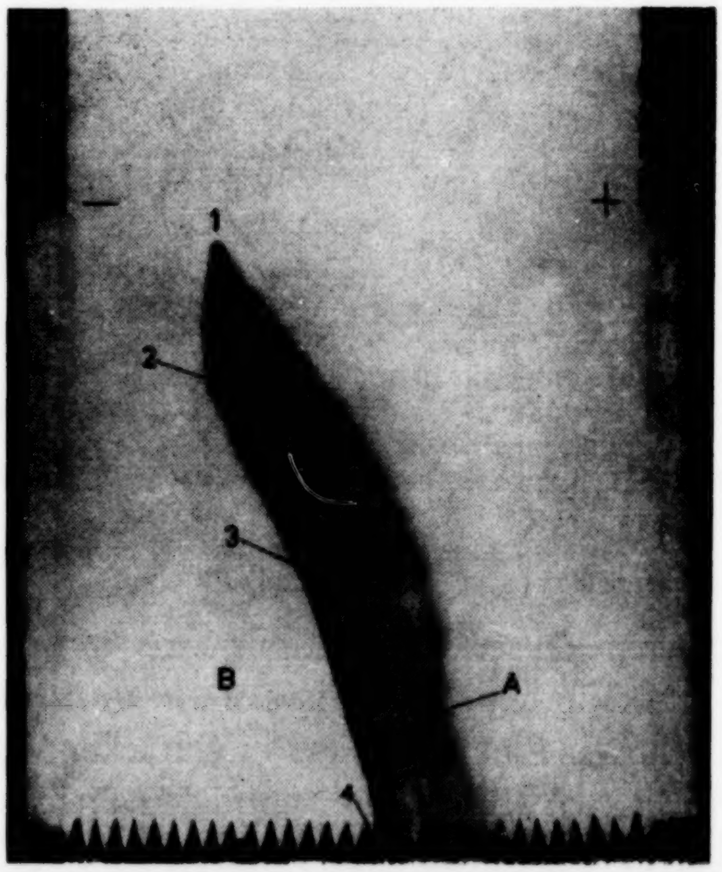

Fig. 47. Lipidogramm der kontinuierlichen Elektrophorese eines normalen menschlichen Serums in Na-Veronal–Na-Acetat-Puffer bei pH 8.6 nach Pučar, Keler und Petek[88]. Filtrierpapier Munktell No. 20/100, Anfärbung mit Sudanschwarz. 1 = Stelle der Zufuhr des Serums; 2 = Stelle der Aufspaltung des γ-Lipoproteinkomplexes in γ'-Lipide und γ-Globuline; 3 = Stelle an der die Komplexbildung des γ'-Lipid–α'_1-Lipid-Komplexes erfolgt, 4 = Abtropfstelle des γ'-Lipid–α'_1-Lipid-Komplexes; B = kaum sichtbare Bahnen der γ- und β-Lipoproteine; A = A-Lipoproteine; 1–2 = Bahn der γ'-Lipoproteine; 2–3 = Bahn der freien γ'-Lipide; 1–3 = Bahn der freien α'_1-Lipide; 3–4 = Bahn des γ'-Lipid–α'_1-Lipid-Komplexes.

Form von zwei geradlinigen, sich kreutzenden Bahnen am Trennbogen auftrugen, ähnlich Fig. 46 links. Der Vorteil dieser Arbeitsweise ist, dass nicht nur in entgegengesetzer Richtung wandernde Substanzen, sondern auch Substanzen, die in gleicher Richtung wandern, geprüft werden können. Die Enzym–Substrat-Bindung von Chymotrypsin und Azocasein wurde auf diese Weise veranschaulicht. Man kann erwarten, dass in vielen Fällen von biochemischer oder allgemein chemischer Bedeutung die Bildung von Additionsverbindungen in Lösungen auf diesem Wege gezeigt werden kann, wie z.B.: Farbstoff–Farbstoff, Eiweiss–Farbstoff, Protein–Nukleinsäure, Antigen–Antikörper, Enzym–Substrat, u.s.w.

Dieses Phänomen haben auch Pučar, Keler und Petek[88] bei der diskontinuierlichen und kontinuierlichen Elektrophorese der Fette von menschlichen Sera beschrieben (Fig. 47). Die durch den Vorgang der Trennung von γ-Globulinen befreiten γ'-Lipide änderten plötzlich die Wanderungsrichtung, und dabei kreuzten sie die Bahn der freien α'_1-Lipiden, um dann bis zur Abtropfstelle gemeinsam mit ihnen zu wandern, vermutlich in Form einer komplexen Verbindung.

(*b*) *Diskontinuierliche oder zweidimensionale elektrochromatographische Trennungen*

Obwohl bereits 1948 Haugaard und Kroner[1] durch Verteilungschromatographie am Trennbogen unter gleichzeitiger Verwendung eines Potentialgefälles senkrecht zur chromatographischen Richtung eine Mischung von 10 Aminosäuren bestehend aus zwei Dikarbonsäuren (Asparaginsäure und Glutaminsäure), zwei basischen Aminosäuren (Lysin und Arginin) und sechs neutralen Aminosäuren (Serin, Glycin, Alanin, Valin, Leucin, und Prolin,) vollkommen voneinander trennten, scheint es doch, dass diese Arbeitsweise bis jetzt kaum aufgenommen und weiterentwickelt wurde. Der Trennbogen war mit einem Phosphatpuffer von pH 6.2 vorpräpariert. Als Lösungsmittel wurde Phenol verwendet. Durch ähnliche Technik trennte Burma[149] Aminosäuregemische bei pH 2.2, 4.0 und 6.0.

Strain und Sullivan[7] trennten diskontinuierlich Metallionen, von denen einige Trennungen hier genannt werden sollen: Ni–Co–Fe–Ag–Cd–Cu in einer Mischung von 0.01 *M* Ammoniumtartrat, 0.005 *M* Dimethylglyoxim und 4 *M* Ammoniak, Hg–Bi–Cd–Pb–Cu in 0.1 *M* Milchsäure, und Ni–Fe–Co–Al in einer Mischung von 0.01 *M* Weinsäure, 0.005 *M* Dimethylglyoxim und 4 *M* Ammoniak. Achiwa[150–152] befasste sich mit der Trennung anorganischer Anionen. Unter anderen wurden folgende Trennungen angegeben[150]: SO_3^{2-}–SO_4^{2-}–CrO_4^{2-}–PO_4^{3-}–AsO_4^{3-} in einer Mischung von 0.0025 *M* $AgNO_3$ und 0.02 *M* Ammonacetat, S^{2-}–AsO_3^{3-}–BO_2^-–$Fe(CN)_6^{4-}$–$Fe(CN)_6^{3-}$ in einer Mischung von 0.005 *M* $Fe_2(SO_4)_3(NH_4)_2SO_4$ und 0.1 *M* Essigsäure, und J^-–Br^-–Cl^-–NCS^-–CN^- in einer Mischung von 0.01 *M* $NiSO_4$ und 0.02 *M* Ammonacetat. Ausserdem wurde auch eine Tabelle zum systematischen qualitativen Analysengang für sämtliche anorganische Anionen zusammengestellt[150]. Pučar und Jakovac[104] trennten diskontinuierlich Mischungen radioaktiver seltener Erden ^{144}Ce–^{160}Tb–^{170}Tm und ^{144}Ce–^{152}Eu–^{169}Yb in 0.05 *M* Milchsäure (Fig. 48). Pučar[36] trennte die Farbstoffmischung Tropäolin O–Fluorescein–Chlorphenolrot–Rosolsäure–Chrysoidin Y–Nitranilin in einer Boratpufferlösung bei pH 8.6 vollkommen in Komponenten (vergleiche Fig. 36). McDonald, Bermes und Shepherd[42] trennten durch zentrifugalbeschleunigte zweidimensionale Elektrochromatographie bei pH 8.0 in 14 Minuten die Farbstoffmischung Anilinrot–Bromphenolblau–Methylorange. Durch dieselbe Technik trennten McDonald, Ribeiro und Banaszak[45] im Phosphatpuffer bei pH 6.4 und Ionenstärke 0.008 in 15 Minuten die Aminosäuremischung Arginin–Histidin–Prolin–Tyrosin–Asparaginsäure. Im Veronalpuffer bei pH 8.6 und Ionenstärke 0.01 trennten in 15 Minuten McDonald und Ribeiro[44] die Mischung Glycin–Lysin–Glutaminsäure.

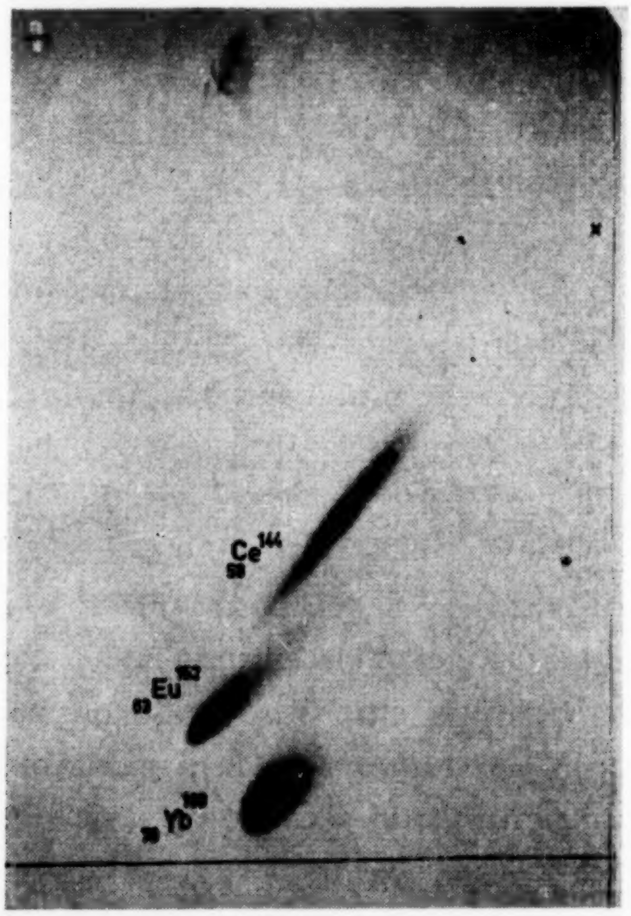

Fig. 48. Autoradiographie einer zweidimensionalen elektrochromatographischen Trennung von ^{144}Ce–^{152}Eu–^{169}Yb in 0.05 *M* Milchsäure nach Pučar und Jakovac[104]. Papier Munktell No. 20/250. Die horizontale gestrichelte Linie gibt die Front wieder, an der sich Zonen befinden würden die am Filtrierpapier nicht adsorbiert werden.

Pučar und Petek[87] trennten Proteine normaler und pathologischer menschlicher Sera (Fig. 49). Bei pH 8.6 zeigen die Eiweissfraktionen eine sehr geringe Adsorption an der Cellulose des Filtrierpapiers, die dem Adsorptions R_F-Wert von 0.95 entspricht, wärend nur α_2-Globuline einen etwas kleineren R_F-Wert (0.89) aufweisen. Alle Eiweissfraktionen normaler und pathologischer Sera hinterlassen auf dem Filtrierpapier sichtbare Bahnen, die aus auf dem Filtrierpapier adsorptiv zurückgehaltenen Restproteinen bestehen. Zwischenbahnen, die den zwischen den Beweglichkeiten der Haupteiweissfraktionen liegenden Beweglichkeiten entsprechen würden, konnten nicht festgestellt werden. Nach Pučar, Keler und Petek[88] wird durch die gleiche Methode die Trennung und Unterscheidung der nichtadsorbierten Lipoproteine des Serums von den am Filtrierpapier adsorbierten Lipiden ermöglicht. Auf Grund des chromatographischen und elektrophoretischen Verhaltens wurden die Lipide des

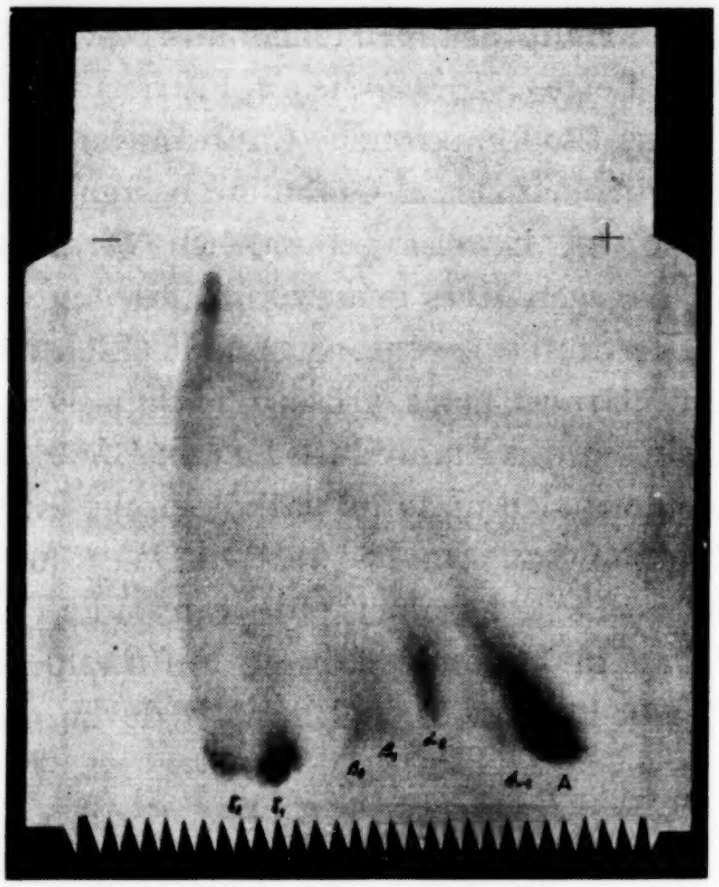

Fig. 49. Zweidimensionales Elektrochromatogramm eines pathologischen menschlichen Serums (multiples Myelom) in Veronal–Na-Acetat-Puffer bei pH 8.6 nach Pučar und Petek[87]. Färbung auf Proteine. Von rechts nach links: Albumine, α_1-, α_2-, β_1-, β_2-, γ_1-, γ_2-Globuline. Die aus Restprotein bestehenden Bahnen sind deutlich sichtbar.

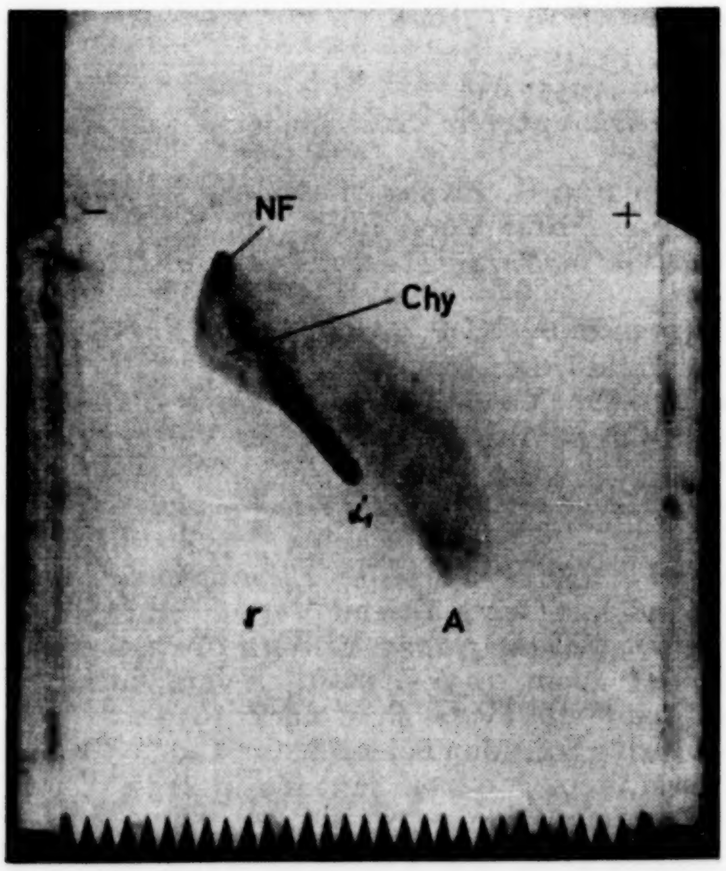

Fig. 50. Zweidimensionales Elektrochromatogramm eines normalen menschlichen Serums im Na-Veronal–Na-Acetat-Puffer bei pH 8.6 nach Pučar, Keler und Petek[88]. NF = Neutralfette; Chy = Chylomikronen; $\alpha'_1 = \alpha'_1$-Lipide; A = A-Lipoproteine; $\gamma = \gamma$-Lipoproteine.

menschlichen Serums in drei Hauptgruppen eingeteilt (Fig. 50): (1) die am Startpunkt fest adsorbierten neutralen Fette (NF), (2) die am Filtrierpapier bedeutend adsorbierbaren freien Lipide, die aus Chylomikronen (Chy) und einer Lipidfraktion mit der elektrophoretischen Beweglichkeit der α'-Globuline bestehen (α'_1), und (3) die praktisch nicht adsorbierbaren, an Eiweiss gebundenen wirklichen Lipoproteine. Die Kohlenhydrate wandern dagegen stets gemeinsam mit den entprechenden Proteinfraktionen und lassen sich durch zweidimensionale Elektrochromatographie von ihnen nicht trennen. Auf Grund eines grösseren klinischen Materials teilen entsprechend den Verschiebungen des Lipidbildes Keler-Bačoka, Pučar und Petek[89] menschliche Sera in einen normalen und fünf pathologische Lipidtypen. Anschliessend studierten Benaš, Keler-Bačoka und Pučar[144] die Wirkung des Heparins und des Protaminsulphats auf menschliche Serum-Lipide während alimentärer Hyperlipemie. Die Wirkung von Dextran in kolloider Lösung auf Lipide der menschlichen Sera wurden von Pučar, Keler-Bačoka und Benaš[145] durch dieselbe Technik veranschaulicht.

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PAPER CHROMATOGRAPHIC ANALYSIS IN CONNECTION WITH THE FORMATION OF TELLURIUM (VI) COMPOUNDS

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When making Te(VI) compounds from Te(IV) it was found necessary to follow the progress of the oxidation process as well as to establish its completeness and finally to examine the stability conditions of the tellurate thus produced. A simple and rapid analytical method was required for this purpose and the simultaneous detection of Te(IV) and Te(VI) by means of paper chromatography seemed the most convenient technique[1].

EXPERIMENTAL

Chemicals. Na_2TeO_3 p.a. Merck; TeO_2 and H_6TeO_6 p.a. B.D.H.

Solvent. *n*-Butanol saturated with 3 *N* HCl.

Detecting agents.

(a) Sn(II) solution: 56.5 g $SnCl_2 \cdot 2H_2O$ dissolved in 80 ml conc. hydrochloric acid and made up with water to 1 l. In order to prevent the oxidation of Sn(II) some tin metal was added to the solution.

(b) KI solution: 20 g KI (p.a. Reanal) dissolved in water, after addition of 50 ml boiled (1:1) hydrochloric acid; 100 ml of solution is prepared by adding water.

Paper. Schleicher and Schüll 2043A.

Method

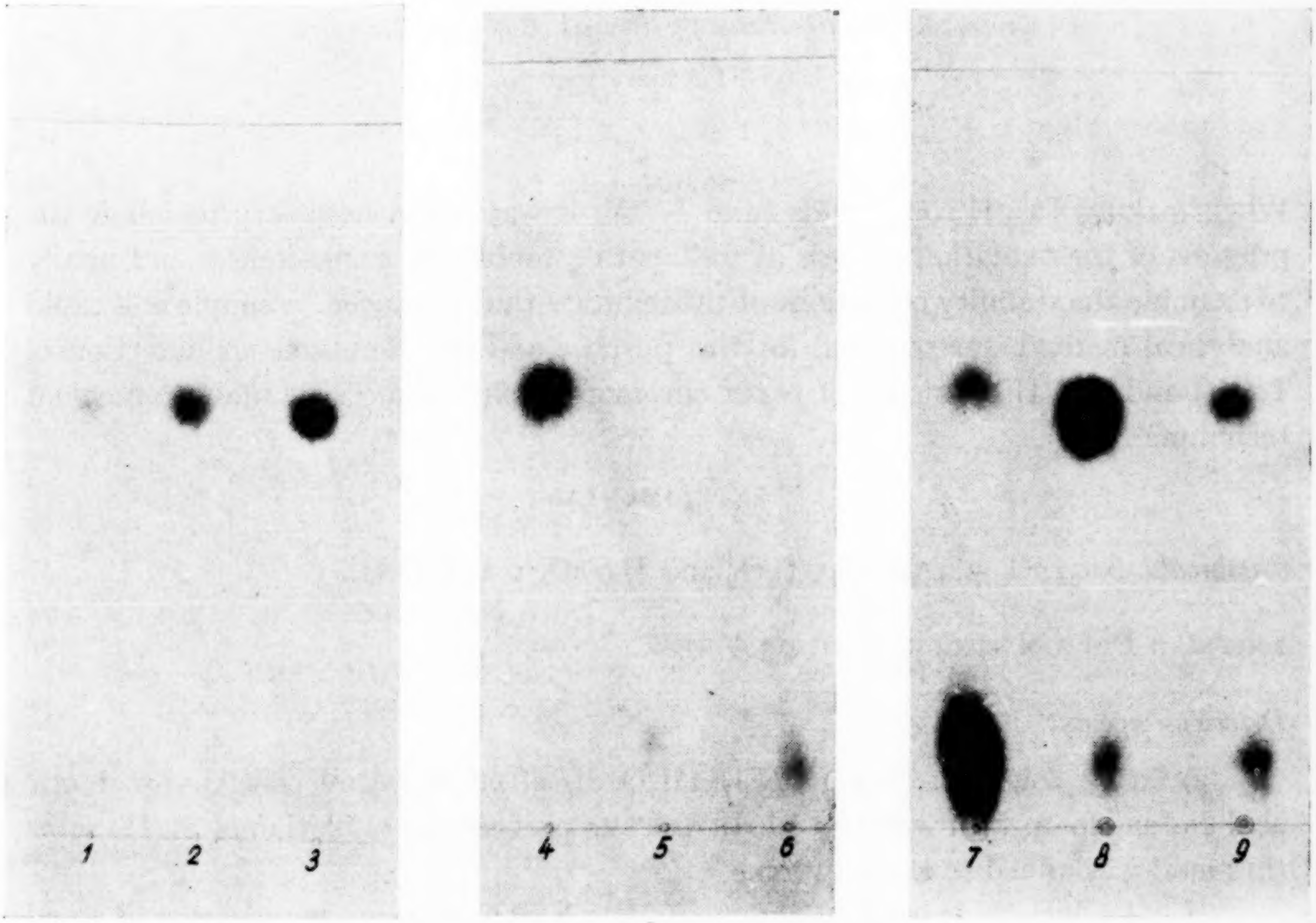
The papers were developed by the ascending method in a covered glass cylinder at 20°. The solution was spotted onto the paper by a micropipette and was dried by a current of cold air—especially when examining the acidic Te(VI) solution. The solvent travelled 20 cm in about 15 hours. After removing the paper from the solvent it was dried by a current of warm air or by suspending it at a suitable distance from an infrared radiator. By viewing the still wet chromatogram under U.V. light, the sodium chlorate which may be present, could be detected as a dark spot. The chromatograms are best shown up by spraying with a stannous chloride solution. By using an acidic potassium iodide solution compounds of Te(IV) and Te(VI) as well as chlorate ions can be located. The sodium ions are shown up by spraying with zinc uranyl acetate and identified by the yellowish green fluorescence appearing under U.V. light.

RESULTS

Figs. 1a and b are photographs of chromatograms of increasing amounts of Te(IV) and Te(VI) compounds.

In our experiments it was found that 1 μg of Te(IV) and 2 μg of Te(VI) can be accurately detected and even less than 1 μg of Te(IV) can be identified.

From Fig. 1c it can be seen that when Te(IV) and Te(VI) are present together 4.8 μg Te(IV) and 100 μg Te(VI) or vice versa can easily be identified.



a b c

Figs. 1a, b and c. Chromatograms of various quantities of Te(IV) and Te(VI) ions. (1) 1 μg Te(IV); R_F = 0.60. (2) 5 μg Te(IV); R_F = 0.60. (3) 9.2 μg Te(IV); R_F = 0.60. (4) 18.8 μg Te(IV); R_F = 0.57. (5) 1.9 μg Te(VI); R_F = 0.11. (6) 2.9 μg Te(VI); R_F = 0.08. (7) 4.8 μg Te(IV), 112 μg Te(VI). (8) 4.8 μg Te(VI), 93.2 μg Te(IV). (9) 4.8 μg Te(IV), 4.8 μg Te(VI).

In Fig. 2 the chromatograms of the following solutions are shown: (a) TeO_2 dissolved in 1 + 3 HCl chlorinated for 5 min. (b) TeO_2 dissolved in 2 *N* NaOH chlorinated for 5 min. (c) Na_2TeO_3 dissolved in HCl. In chromatogram (b) the $NaClO_3$ moving with the solvent front was identified by spotting with $NaClO_3$ solution and detecting with KI solution.

In the chromatograms of Te(IV) compounds it can be observed that the pH of the spotted solution has some effect on the R_F value of these compounds. This effect manifests itself in the fact that higher R_F values are obtained by spotting aqueous or acidic solutions than with alkaline solutions. On adding some alkali to the spotted Te(IV), the R_F value decreases with increasing amounts of sodium hydroxide. In

Fig. 3 the maximum of this effect observed in our experiments is shown. The R_F values 0.55 and 0.53 measured in an acidic solution containing 44 μg Na_2TeO_3 and in an aqueous solution of 107 μg Na_2TeO_3, respectively, decreased to 0.33 when a 2 *N* NaOH solution containing about 59 μg sodium tellurite was run. We assume that this is caused not only by the excess of sodium cations, but also by the fact that the equilibrium $Te^{4+} + 3OH^- \rightleftharpoons TeO_3^{2-} + 3H^+$ as well as the dissociation of the tellurous acid and the hydrolysis of sodium tellurite are affected by the hydroxyl ions. Thus in an acidic solution the Te(IV) cation or the poorly dissociated tellurous acid (H_2TeO_3) move faster than the tellurite anion travelling together with the sodium cation.

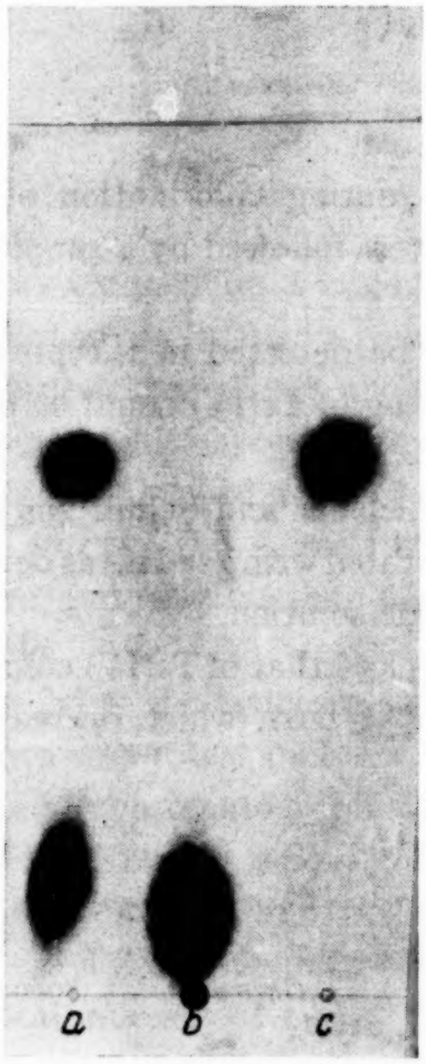


Fig. 2. (a) 39.3 μg TeO_2; HCl + Cl_2; $R_F = 0.13$; 0.61. (b) 64.9 μg TeO_2; NaOH + Cl_2; $R_F = 0.094$; 0.95. (c) 58.8 μg Na_2TeO_3; HCl; $R_F = 0.61$.

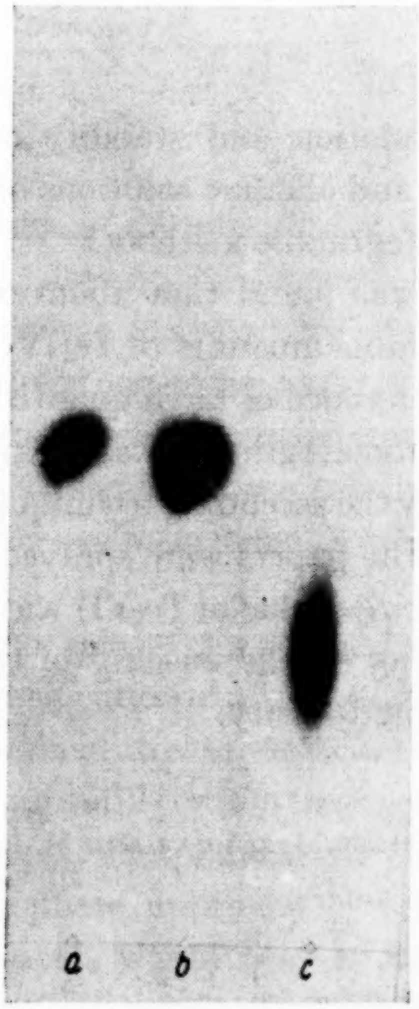


Fig. 3. (a) 44.2 μg Na_2TeO_3; 1 + 3 HCl; $R_F = 0.55$. (b) 107.5 μg Na_2TeO_3; H_2O; $R_F = 0.53$. (c) 58.8 μg Na_2TeO_3; 2 *N* NaOH; $R_F = 0.33$.

In our experiments it was found that it is not only by boiling with hydrochloric acid that Te(VI) compounds are reduced to Te(IV). This process, which at room temperature is very slow, is increased considerably if a solution of Te(VI) in HCl spotted on filter paper is dried by a warm current of air, or if such a solution is carefully evaporated. It is known that Te(IV) is readily oxidized to Te(VI) in neutral, acidic or alkaline systems by the addition of any of the numerous oxidizing agents, for instance chlorine gas. In alkaline systems the oxidation proceeds more rapidly and the Te(VI) compound thus produced is stable. When such solutions are chromatographed the sodium chlorate produced travels together with the solvent front (R_F = 0.95 – 0.97, see (b) in Fig. 2), the sodium chloride leaves the base line (R_F = 0.1), Te(IV) and Te(VI) (R_F = 0.51 – 0.61 and 0.08 – 0.15, respectively) travel according to speeds which are in good agreement with the values given in the literature[1] and thus the two tellurium compounds with different oxidation states can be well detected even when they are present together.

SUMMARY

The formation and stability of Te(VI) compounds during chlorination of acidic, neutral and alkaline solutions of Te(IV) compounds were followed by a simple paper-chromatographic method.

It was found that about 2 μg of Te(VI) could be detected in the presence of considerable amounts of Te(IV) and that less than 1 μg of Te(IV) could be detected in the presence of large quantities of Te(VI).

Chromatography was carried out at 20° on Schleicher and Schüll 2043A filter paper by the ascending technique, using *n*-butanol saturated with 3 *N* HCl as developing agent. The papers were sprayed with stannous chloride solution.

The R_F value of Te(VI) was found to be 0.08–0.15 and that of Te(IV) compounds, depending on the basicity of the spotted solution, 0.51–0.61, which decreases with increasing basicity.

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THE ANALYSIS OF PLASTICIZERS BY MICRO-ADSORPTION CHROMATOGRAPHY

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Plasticizers are used on a large scale in the manufacture of various products of the plastics industry. In many countries the plasticizers, used in the manufacture of plastics for packing cheese and of other sheets intended as food-packing materials, may only contain components that are known to be entirely non-toxic. Some countries have already published a list of plasticizers that are considered as entirely harmless to health (for the U.S.A. see LEHMAN[1]). For a reliable control of these regulations a convenient and rapid method of analysis for the plasticizers mentioned in these lists is desirable.

Some types of plasticizers such as phosphates and phthalates can be distinguished by qualitative spot test analysis. A general system of classification of all plasticizers can be obtained by chromatographic analysis of the acids obtained after saponification. This classification, however, is not sufficient, because these groups still contain toxic as well as non-toxic plasticizers, *e.g.* toxic butyl glycolate and tricresyl phosphate as well as ethyl phthalyl ethyl glycolate and 2-ethylhexyl diphenyl phosphate, approved by LEHMAN.

A total analysis of all the groups bound by the acids would be very difficult.

CACHIA[2] succeeded in identifying the components of a mixture of plasticizers by combining a separation by means of column chromatography with a consecutive infra-red analysis of the various fractions.

A separation of some plasticizers by gas–liquid chromatography at very high temperatures has recently been reported.

Paper chromatography of plasticizers has found little application. BURNS[3] made use of paper impregnated with a polyvinyl chloride–water dispersion for the separation of some homologous series of phthalates, phosphates etc., using potassium permanganate or a universal indicator as coloring reagents. With this method stronger as well as more specific reagents could not be used.

A quick and very simple analysis of an unknown mixture of plasticizers can be obtained by applying the so-called two-dimensional *micro-adsorption chromatography* on chromatoplates. This method was especially worked out in 1951 by KIRCHNER[4] and later was standardized by STAHL[5]. In micro-adsorption chromatography it is possible, in contrast with paper chromatography, to use various strong reagents for identifying the spots, *e.g.* chemicals like concentrated sulphuric acid etc.

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Some of the spot test color reactions, modified by us, are in some cases specific for various plasticizers (see Table II).

Another advantage is the quick and simple way in which the analysis can be carried out, especially if compared with the time-consuming separations by column chromatography.

Only those plasticizers mentioned in the already published lists should be used in the manufacture of food-packing materials.

We have tested some of the plasticizers, approved by LEHMAN, with micro-adsorption chromatography (see Table I). Some not quite harmless plasticizers, frequently used in other branches of the plastics industry, *e.g.* dibutyl phthalate, tricresyl phosphate and triphenyl phosphate are also mentioned in this table.

EXPERIMENTAL

The investigation was carried out using the standardized method for micro-adsorption chromatography, as developed by STAHL. With an apparatus, specially designed for this purpose, an adsorbent is poured out on a glass plate in a film of approximately

TABLE I

RELATIVE R_A VALUES OF SOME PLASTICIZERS WITH MICRO-ADSORPTION CHROMATOGRAPHY IN THREE SOLVENT MIXTURES

Identification with 0.005 % Ultraphor in U.V. light.

R_A value = R_F value with reference to dibutyl sebacate.

Plasticizer	*R_A value in the solvent mixtures*		
	Iso-octane + 10 % ethyl acetate	*Benzene + 5 % ethyl acetate*	*Dibutyl ether + 20 % hexane*
1. Triacetin	0.18	0.34	0.17
2. Ethyl phthalyl ethyl glycolate	0.22	0.66	0.30
3. Acetyl triethyl citrate	0.26	0.51	0.29
4. Triphenyl phosphate	0.33	0.80	0.50
5. Tricresyl phosphate	0.42	0.86	0.69
6. Butyl phthalyl butyl glycolate	0.43	0.90	0.65
7. 2-Ethylhexyl diphenyl phosphate	0.46	0.77	0.58
8. Diethyl phthalate	0.51	0.79	0.60
9. Acetyl tributyl citrate	0.53	0.85	0.70
10. Di-*n*-butyl phthalate	0.74	1.03	0.84
11. Di-isobutyl adipate	0.83	0.86	0.85
12. Dibutyl sebacate	≡ 1.00	≡ 1.00	≡ 1.00
13. Dinonyl phthalate	1.01	1.18	1.14
14. Di-2-ethylhexyl phthalate	1.14	1.16	1.15
15. Butyl stearate	1.61	1.23	1.28
16. Paraflex G 62 (epoxidized natural glycerides)	0.09 0.15 0.25 0.33 0.60	0.06 0.13 0.20 0.26 0.40 0.47 0.65 0.72 1.04	0.06 0.14 0.28 0.42

0.3 mm thickness. The adsorbing material consists of a mixture of silicagel and plaster of Paris ("Siligacel G", manufactured by Merck).

The plasticizer or the residue obtained by extraction of a food-packing material is dissolved in diethyl ether (5 %). 10 mm³ of this solution is placed at the starting point on a chromatoplate.

For the purpose of indicating all types of plasticizers, the silicagel G–water mixture was provided with 0.005 % of a water-soluble fluorescence indicator, *e.g.* Ultraphor*. On irradiation with ultra-violet light (365 mμ) all plasticizers become clearly visible as fluorescent spots or, in the case of phthalates, as dark spots.

The following solvent mixtures are preferably used: iso-octane + 10 % ethyl acetate, benzene + 5 % ethyl acetate and dibutyl ether + 20 % hexane.

The R_F values of the plasticizers are calculated with reference to dibutyl sebacate. These relative, so-called R_A values (see Table I) show a sufficient reproducibility. The variations go up to 0.06.

By using the solvent mixture iso-octane + 10 % ethyl acetate the components indicated by the numbers 1, 3, 4, 6, 9, 10, 11, 12, 14 and 15 in Table I are completely separated (see Fig. 1). A complete separation of the pairs: 2–3, 8–9, 5–8, 7–8, 6–7 and 6–8 in this table, which cannot be separated in the system iso-octane + 10 % ethyl acetate, can be obtained in one of the other two systems. Only the "critical pairs" with the numbers 5–6, 5–7 and 7–9 (see Table I) are not sufficiently separated in any of the systems and have to be identified by means of the specific reagents mentioned in Table II.

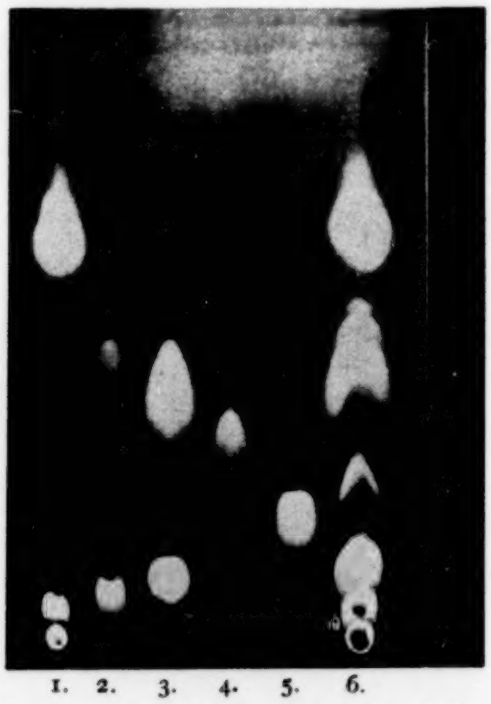

Fig. 1. Separation of 10 plasticizers on a chromatoplate. Solvent mixture: iso-octane + 10 % ethyl acetate. Identification with 0.005 % Ultraphor in U.V. light (365 mμ). (1) Triacetine + butyl stearate. (2) Acetyl triethyl citrate + di-2-ethylhexyl phthalate. (3) Triphenyl phosphate + dibutyl sebacate. (4) Butyl phthalyl butyl glycolate + di-isobutyl adipate. (5) Acetyl tributyl citrate + di-*n*-butyl phthalate. (6) Mixture of all these plasticizers.

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* Ultraphor of the Badische Anilin & Soda Fabrik, according to R. TER HEIDE (personal communication).

TABLE II

COLOR REACTIONS OF SOME PLASTICIZERS (500 μg) ON CHROMATOPLATES

Plasticizer	Color reaction*								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
1. Triacetin	yellow	purple	yellow-green	—	blue-black	—	purple-red	—	—
2. Ethyl phthalyl ethyl glycolate	—	yellow	purple	—	—	—	—	—	—
3. Acetyl triethyl citrate	—	—	—	+	blue	—	± yellow	—	yellow
4. Triphenyl phosphate	orange-yellow	—	—	—	red	blue	yellow	—	orange-purple
5. Tricresyl phosphate	red	—	—	—	red	blue	yellow	—	orange-purple
6. Butyl phthalyl butyl glycolate	yellow	yellow	purple	—	—	—	—	—	—
7. 2-Ethylhexyl diphenyl phosphate	blue	purple	green	—	blue	blue	yellow	purple brown	orange-purple
8. Diethyl phthalate	yellow	yellow	—	—	—	—	purple-red	—	—
9. Acetyl tributyl citrate	± yellow	—	—	+	blue	—	±	± pink	yellow
10. Di-*n*-butyl phthalate	± yellow	yellow	blue	—	—	—	± yellow	—	—
11. Di-isobutyl adipate	± yellow	—	± yellow	—	green-blue	—	± yellow	± pink	—
12. Dibutyl sebacate	± yellow	—	± blue	—	—	—	± yellow	±	—
13. Dinonyl phthalate	blue	± orange	brown-red	—	blue	—	—	purple-brown	—
14. Di-2-ethylhexyl phthalate	blue	± orange	green	—	black	—	± yellow	purple-brown	—
15. Butyl stearate	blue	—	—	—	—	—	yellow	brown-red	—
16. Paraflex G 62, $R_F \sim 0.00$	blue	± brown	brown	+	black	—	yellow	brown	—

* Color reactions in which only a faint color is obtained are indicated as ±.

It is often desirable to make also a few two-dimensional chromatograms when carrying out the separation of the pairs of plasticizers mentioned above.

Some plasticizers, such as Paraflex G 62, originating from natural products are of a more complex nature. When analysing these plasticizers by micro-adsorption chromatography we found a series of spots (Table I, No. 16). The presence of these plasticizers can be demonstrated by specific color reactions, but because of the great number of spots the identification of other plasticizers is severely hindered.

Table II contains a survey of some more or less specific color reactions. The procedure of these spot test color reactions is as follows:

1. Spray with a solution of phosphomolybdic acid (10 % in ethanol). Heat approx. 20 min at 100°.

2. Spray with a solution of resorcinol (20 % in ethanol; add some $ZnCl_2$). Heat 10 min at 150°. Spray with 4 N H_2SO_4. Heat approx. 20 min at 120°. Spray with 40 % KOH.

3. Spray with a solution of thymol (20 % in ethanol). Heat 10 min at 90°. Spray with 4 N H_2SO_4. Heat approx. 10–15 min at 120°.

4. Spray with a 1 N alcoholic KOH solution. Heat 15 min at 60°. Only the citrates become visible as yellow spots. Spray with a 50 % solution of urea. The citrates are visible in U.V. light (365 $m\mu$) as strong fluorescent spots.

5. Spray with a solution of vanillin (20 % in ethanol). Heat 10 min at 80°. Spray with 4 N H_2SO_4. Heat approx. 30 min at 110°.

6. Spray with a solution of 2,6-dichloroquinone-chloroimide (2 % in ethanol). Spray after 2–3 h with 2 % borax.

7. Spray with an alkaline $KMnO_4$ solution (a mixture 1:1 of 1 % $KMnO_4$ and 2 % Na_2CO_3). The colors shown after 1–2 h are noted.

8. Spray with a mixture of acetic anhydride–50 % H_2SO_4 (1:2). Heat approx. 10 min. at 80°.

9. Spray with a 0.5 N ethanolic solution of KOH. Heat 15 min at 60°. Spray with a diazonium reagent (prepared from 0.8 g *p*-nitraniline in 250 ml water and 20 ml 25 % HCl; a 5 % $NaNO_2$ solution is added till the reagent mixture is entirely colorless).

CONCLUSION

By determining the relative R_A values of the solvent systems mentioned in Table I and by using the color reactions mentioned in Table II, the components of a mixture of plasticizers can in most cases be identified.

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SUMMARY

Various plasticizers can be separated by micro-adsorption chromatography, according to STAHL's method.

The R_F values of some of the plasticizers, approved in the U.S.A. for application in food-packing materials, have been determined in three solvent systems.

Some color reactions, more or less specific for various plasticizers, have been used.

A mixture of unknown plasticizers can be identified by the R_F values in three solvent systems and by the colors obtained with various staining methods.

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A CHROMATOGRAPHIC PROCEDURE FOR SEPARATING CHLORPROMAZINE DERIVATIVES

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In the course of an investigation of the products of the metabolism of chlorpromazine in the whole animal, the need arose for a chromatographic procedure for separating the derivatives of chlorpromazine that might result from oxidative attack on the molecule at the sulfur position, demethylative attack at the dimethylamino position or both. The structures that could result from either or both modes of attack are shown in Fig. 1. Although a number of chromatographic procedures have been reported for separating chlorpromazine from related compounds[1-5], none of them appeared

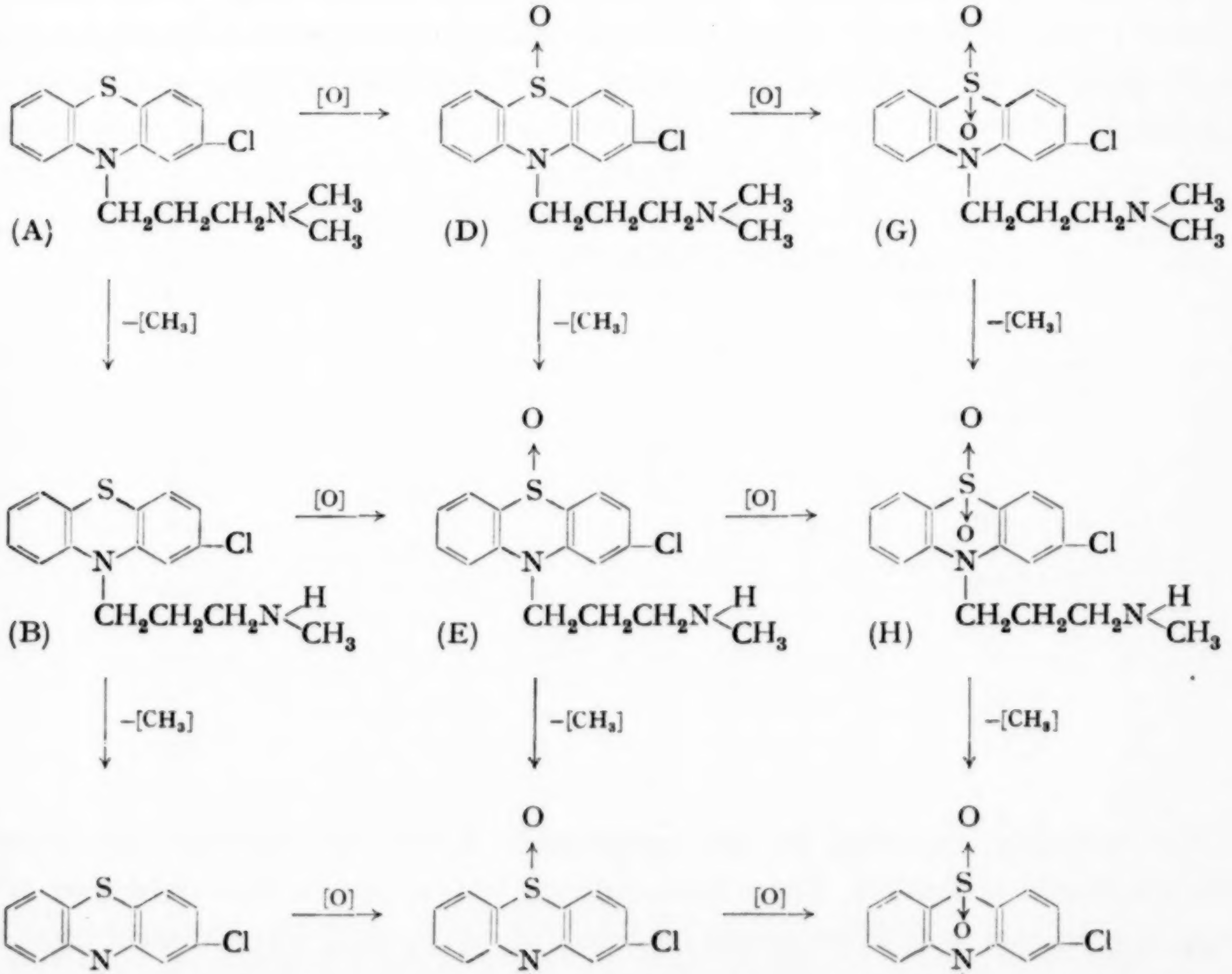

Fig. 1. Possible oxidation and demethylation products of chlorpromazine.

capable of resolving all or even most of the compounds in this series. The successful development of such a procedure is the basis for this communication.

As a result of testing a number of solvent combinations, one was found that appeared capable of effecting the desired resolution. This was a 3:1:4:2 by volume mixture of 1,2-dichloroethane–benzene–formic acid (88%)–water. It was used in a conventional manner, that is, with the organic layer as the moving phase and the aqueous layer as the standing phase. Since the phases did not differ much in density, it was often advantageous to warm the mixture slightly to speed phase separation. It was also convenient to test each layer for miscibility with benzene to determine with certainty which was the organic phase.

Since most of the compounds moved rather slowly in this system, descending development was used with excess solvent running off the serrated lower edge of the paper. To determine optimum development times, strips of Whatman 3 MM* paper were spotted with 20 γ of each of the compounds, equilibrated overnight (16 h) in solvent-saturated atmosphere and developed in descending fashion at room temperature (25°) for 6, 12, 24 and 36 h. The strips were then examined under short wavelength U.V. light (low pressure mercury vapor). All of the compounds showed up sharply as fluorescent spots on a dark background. The sulfides appeared greenish-blue, the sulfoxides reddish-blue, and the sulfones pale-blue. The strips were also sprayed with the MUNIER AND MACHEBOEUF iodoplatinate reagent for alkaloids[6] (acidified 1:1000 with HCl), which produced bluish-purple spots on a pale pink background. Both of the detection procedures were sensitive to 1–2 γ of each of the compounds.

TABLE I

EFFECT OF PROLONGED DEVELOPMENT ON SEPARATION OF SOME CHLORPROMAZINE DERIVATIVES IN DESCENDING SYSTEM

	Distance moved (cm)			
Development time (h)	*6*	*12*	*24*	*36*
Compound				
A	26.1	45	off (> 46)	off
B	18.1	31	off	off
C	5.8	10.7	19.5	32.0
D	3.5	5.4	10.5	15.9
E	1.6	3.0	6.0	9.7
F	0.25	0.6	1.3	2.4
G	5.9	8.8	14.7	22.7
H	2.3	4.0	7.0	11.7
I	0.5	0.75	1.6	3.2

The distances travelled by the compounds during the different development times are shown in Table I. These data indicate that a satisfactory resolution of the compounds in each oxidation group (sulfide, sulfoxide, etc.) was obtained after 6 h, although there was still considerable overlapping of compounds in adjacent groups.

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* Whatman No. 1 paper also provided satisfactory separations with this system, however, development times were longer and resolutions somewhat poorer than with 3 MM paper.

Even better resolution of compounds in each oxidation group was obtained after development for 12 h, at which time all but one or two pairs (E,H; F,I) of very slow moving compounds in adjacent groups were well separated. After 24 h the two fastest moving compounds (A, B) had moved off the paper, but all of the remaining compounds were well separated except for the two slowest moving (F, I), which still overlapped somewhat. Development for 36 h was required to adequately separate this pair.

From these data it appeared that a reasonably complete resolution of the compounds in this series could be obtained by running two chromatograms, one for 12 h to keep all of the compounds on the paper and a second for 24 or 36 h to separate the slower moving compounds better. Indeed, when urine extracts from animals fed chlorpromazine were chromatographed by this procedure for 12 and 24 h, very good separations of the derivatives present were obtained (to be reported later).

In an attempt to improve the resolution of the slower moving compounds and still keep the faster moving ones on the paper, a modification* of the GIRI AND RAO[7] circular-development technique was tested. Using the same paper, solvent system and equilibrium time as with the descending system, it was indeed found possible to obtain sharper and better resolved bands by circular development than by conventional descending development. However, since it was not practical to develop circular chromatograms continuously for longer than it took for the solvent front to reach the edge of the paper (about 3 h), it was necessary to resort to repetitive development to resolve the slower moving compounds. That is, each time the solvent front reached a pre-set mark (15 cm from the origin), the paper sheet was removed, dried in air (in subdued light) for about 15 min, re-equilibrated for 1–2 h and developed again to

TABLE II

APPARENT† R_F VALUES OF SOME CHLORPROMAZINE DERIVATIVES AFTER REPEATED DEVELOPMENT IN CIRCULAR SYSTEM

	Apparent† R_F value			
Number of developments	*1*	*2*	*3*	*4*
Compound				
A	0.55	0.76	0.81	0.86
B	0.41	0.62	0.69	0.74
C	0.17	0.34	0.42	0.50
D	0.10	0.20	0.30	0.39
E	0.06	0.14	0.21	0.28
F	0.02	0.04	0.08	0.13
G	0.15	0.26	0.36	0.45
H	0.08	0.16	0.25	0.33
I	0.02	0.03	0.06	0.09

† Distance from origin to band/distance from origin to pre-set solvent front, regardless of number of developments.

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* We used cotton cord instead of folded filter paper for wicks.

mark. It was generally convenient to do two developments a day, and if more were required, to equilibrate overnight before starting the third.

The apparent R_F values of the various compounds developed once, twice, three, and four times by this procedure are listed in Table II. These values indicate that all of the sulfides and some of the sulfoxides and sulfones were resolved by a single development, although many of the slower moving sulfoxides and sulfones still overlapped badly. Double development appeared to separate all of the compounds satisfactorily except for two pairs E, H and F, I. The latter pair did not separate well even with triple development, although all other combinations were cleanly separated. A fourth development effected a practical separation of all compounds in the series and still retained all of them on the sheet.

Although some of the compounds do not appear to differ very much in R_F value, this does not mean that the compounds were not adequately separated. As can be seen in Fig. 2, most of the bands remained rather narrow even after replicate development and so did not tend to overlap adjacent bands. Furthermore, it was generally possible to distinguish closely adjacent bands by their different appearances under ultraviolet light or after spraying with the iodoplatinate reagent.

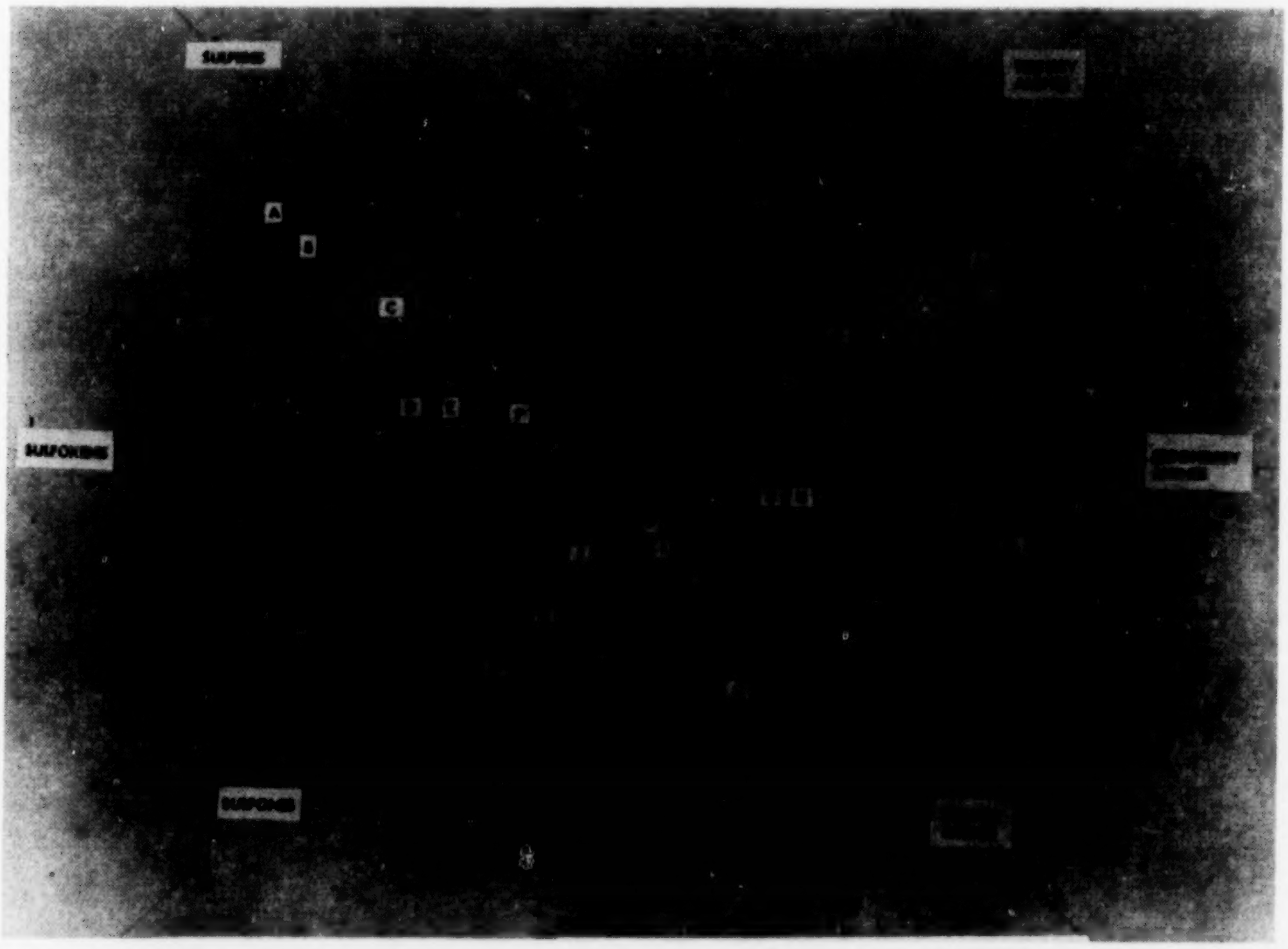

Fig. 2. Separation of 9-chlorpromazine derivatives on Whatman 3 MM paper by triple circular-development with 1,2-dichloroethane–benzene–formic acid–water (3:1:4:2).

The circular procedure had already proven valuable in the separation and identification of the metabolites of chlorpromazine present in the urine of chlorpromazine-treated animals. These results will be reported later.

ACKNOWLEDGEMENTS

We wish to thank Dr. T. H. LIN and Mr. L. REYNOLDS of these Laboratories for suggesting the problem and providing some of the samples. We should also like to acknowledge the technical assistance of Miss T. SAARSE.

SUMMARY

Descending and circular procedures are described for separating the nine derivatives of chlorpromazine that could result from sulfide oxidation, N-demethylation, or both. The solvent system used was a 3:1:4:2 mixture of dichloroethane, benzene, formic acid, and water.

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THE USE OF TRIETHYLENE GLYCOL IN THE PAPER CHROMATOGRAPHY OF STEROIDS

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In the paper chromatography of steroids Zaffaroni's systems with a stationary relatively polar organic phase are routinely used. In addition to formamide and dimethylformamide, glycols or their ethers are most frequently used for this purpose, particularly propylene glycol, ethylene glycol, butane-1,3-diol, glycerol, ethylene glycol monoethyl ether (cellosolve), ethylene glycol monophenyl ether (phenylcellosolve), diethylene glycol monoethyl ether (carbitol), etc.[1].

As regards its properties, triethylene glycol (TEG) occupies an intermediate position between simple glycols and their ethers. It was recently recommended as the stationary phase in paper chromatography of 17-ketosteroids[2]; its use is, however, more universal.

EXPERIMENTAL

Steroids in chloroform solution were applied to sheets of Whatman No. 1 paper (15 × 48 cm). Subsequently the strips were impregnated by soaking in a 30% solution of triethylene glycol in methanol, blotted between filter paper and exposed to air for 20 minutes to allow the surplus methanol to evaporate. The chromatograms were then transferred to the chromatography tanks, saturated with vapours of the mobile phase, and developed by the descending technique at 18–21°.

When applying repeated development in two different systems the chromatograms were transferred, after the front had run about 20 cm in the first system, into the tank with the second mobile phase, which usually ran some 40 cm.

Ketosteroids were detected by treatment with the Zimmermann reagent (15% methanolic potassium hydroxide and 2% ethanolic *m*-dinitrobenzene, mixed before use in a ratio of 1:1). The spots were developed at 85–95°.

RESULTS AND DISCUSSION

For the impregnation of the paper with triethylene glycol a 30% solution of TEG in methanol was used. Paper thus impregnated contains about 30% by weight of TEG and the rate of flow of the mobile phase is 5–10 cm/h, which allows a relatively rapid separation. The spots are round or only slightly elongated and the separation is satisfactory. The effect of the concentration of the impregnation reagent on the R_F

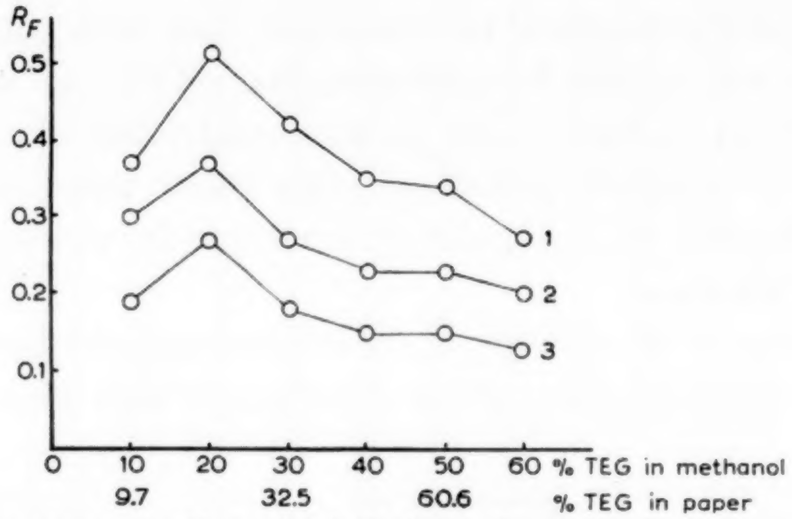

Fig. 1. The effect of the concentration of triethylene glycol in the impregnation solution on the R_F values of some 17-ketosteroids. (1) Androsterone. (2) Epiandrosterone. (3) Dehydroepiandrosterone.

values of dehydroepiandrosterone, etiocholanolone and androsterone in the system petroleum ether/TEG is shown in Fig. 1.

The R_F values of some steroids in systems with stationary triethylene glycol are recorded in Table I.

For the separation of the mixture of neutral urinary 17-ketosteroids repeated development proved very satisfactory; this was carried out first with tetrachloro-

TABLE I

R_F VALUES OF SOME STEROIDS IN SYSTEMS WITH STATIONARY TRIETHYLENE GLYCOL

Steroid	*Mobile phase*				
	Cyclohexane	*Petroleum ether*	*Tetrachloromethane*	*Benzene*	*Toluene*
Cortisol			0.01	0.04	0.02
Aldosterone				0.08	0.05
Cortisone			0.01	0.10	0.06
Corticosterone			0.04	0.25	0.21
11-Desoxycortisone			0.05	0.23	0.20
11β-Hydroxyetiocholanolone	0.01	0.02	0.05	0.16	0.22
11β-Hydroxyandrosterone	0.02	0.03	0.11	0.26	0.27
7-Ketodehydroepiandrosterone	0.02	0.03	0.12	0.36	0.33
Oestrone	0.03	0.04	0.16	0.37	0.37
$\Delta^{4,6}$-Androstadien-17β-ol-3-one	0.04	0.05	0.28	0.50	0.48
Testosterone	0.09	0.12	0.30	0.54	0.55
Adrenosterone	0.07	0.11			
11-Ketoetiocholanolone	0.04	0.06	0.24	0.50	0.62
11-Ketoandrosterone	0.08	0.12	0.35		
17α-Hydroxyprogesterone			0.33	0.60	0.62
Dehydroepiandrosterone	0.16	0.22	0.56	0.60	0.69
Epiandrosterone	0.22	0.31	0.62	0.64	0.68
Etiocholanolone	0.25	0.32	0.63	0.66	0.71
Androsterone	0.34	0.41	0.68	0.68	0.74
Methyltestosterone	0.22	0.26	0.74	0.84	0.90
$\Delta^{4,6}$-Androstadiene-3,17-dione	0.31	0.30	0.73	0.85	0.93
Δ^{4}-Androstene-3,17-dione	0.40	0.50	0.75	0.85	0.93
Androstane-3,17-dione	0.68	0.70	0.85	0.93	0.96
Progesterone	0.64	0.82	0.93		
3β-Chloro-Δ^{5}-androsten-17-one	0.93	0.95			
Pregnane-3,20-dione	0.84	0.90			

methane to about half the length of the sheet and then with petroleum ether to the end of the paper. In the system tetrachloromethane/TEG the more polar 17-ketosteroids, particularly 11-hydroxy- and 11-keto-substituted ones, are separated. In the second run with the mobile petroleum ether phase these steroids change their position only slightly, but the less polar 17-ketosteroids, which were not separated during the first run, separate.

For the calculation of the mobility of the chromatographed substance in repeated development, the relationship derived by PRUSÍKOVÁ[3] was used. This was modified by introducing the value R_{AB} which is the ratio of the distance of the spot after the second development from start (D) to the front of the mobile phase (petroleum ether) after the second development (B) (*cf.* Fig. 2). If the distance of the front of tetrachloromethane is A and the R_F values in the first and second system are R_1 and R_2 resp., then

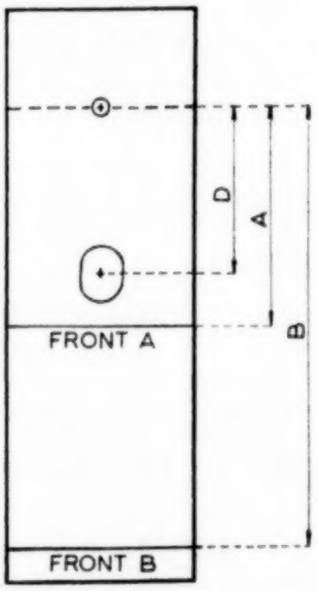

Fig. 2. The concept of the R_{AB} value in repeated development. Front A = distance of the front after the first development. Front B = distance of the front after the second development. D = distance of the spot after the second development.

$$R_{AB} = \frac{D}{B} = \frac{A}{B} \cdot R_1 (1 - R_2) - R_2 \qquad (1)$$

Using equation (1), to which the same limitations apply as mentioned by PRUSÍKOVÁ[3] the R_{AB} values were calculated for six chromatograms selected at random of urinary 17-ketosteroids and were compared with the estimated values. Table II presents a practical test of the validity of equation (1) for calculating R_{AB} values and the suitability of the system for the proper separation of the main urinary 17-ketosteroids on one chromatogram. The results indicate that this chromatographic method can serve well as a basis for the quantitative estimation of urinary 17-ketosteroid fractions, an estimation which is frequently needed in the clinical diagnosis of some endocrine diseases. Another advantage of the TEG systems is the fact that triethylene glycol has practically no influence on the colorimetric estimation of 17-ketosteroids, based on Zimmermann's reaction.

TABLE II

COMPARISON OF CALCULATED AND ESTIMATED R_{AB} VALUES IN REPEATED CHROMATOGRAPHY OF NEUTRAL URINARY 17-KETOSTEROIDS

17-Ketosteroid	*Case*	*J.M.*		*J.M.*		*H.B.*		*J.H.*		*J.G.*		*Z.V.*	
	*Front A cm**	*20.5*		*20.0*		*18.5*		*20.0*		*23.4*		*20.0*	
	*Front B cm***	*36.3*		*38.0*		*36.5*		*37.0*		*35.3*		*38.3*	
	R_{AB}	*calc.*	*est.*	*calc.*	*est.*	*calc.*	*est.*	*calc.*	*est.*	*calc.*	*est.*	*calc.*	*est.*
11-Hydroxyetiocholanolone		0.05	0.04	0.05	0.05	0.04	0.08	0.05	0.05	0.05	0.06	0.05	0.04
11-Hydroxyandrosterone				0.09	0.09	0.08	0.13	0.09	0.10	0.11	0.14	0.09	0.08
11-Ketoetiocholanolone		0.19	0.17	0.18	0.18	0.28	0.19	0.18	0.20	0.21	0.22	0.18	0.20
Dehydroepiandrosterone		0.46	0.43	0.45	0.44	0.44	0.45	0.46	0.49	0.51	0.48	0.45	0.43
Etiocholanolone		0.56	0.48	0.55	0.52	0.54	0.55	0.55	0.57	0.60	0.56	0.54	0.51
Androsterone		0.63	0.56	0.62	0.59	0.61	0.63	0.65	0.65	0.67	0.63	0.62	0.58

* Distance of front A (cm) after first development in system tetrachloromethane/TEG.

** Distance of front B (cm) after second development in system petroleum ether/TEG.

SUMMARY

For the separation of some steroids, especially 17-ketosteroids, systems with stationary triethylene glycol and a mobile phase consisting of petroleum ether, tetrachloromethane, benzene or toluene, are recommended.

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ANALYSE DE MÉLANGES D'ACIDES AMINÉS PAR CHROMATOGRAPHIE GAZEUSE

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Malgré toutes les solutions qui lui ont été proposées, le problème de la séparation et du dosage de mélanges d'acides aminés reste une préoccupation majeure du chimiste organicien. Il n'est donc pas étonnant que les chercheurs s'efforcent d'adapter à ce problème toute nouvelle technique ayant fait ses preuves. C'est actuellement le cas pour la chromatographie gazeuse et cette technique récente a été entre autre utilisée dans ce but en transformant les acides aminés en aldéhydes correspondants[1], en esters méthyliques[2], en aldéhydes puis combustion[3, 4], en esters N-acétyl butyliques[5]. D'autres transformations ont encore été proposées[6-8]. En ce qui concerne le groupe des acides α-monoaminés monocarboxylés nous avons montré dans des travaux antérieurs[9, 10] que la réaction d'un mélange d'HCl et d'HNO_3 concentrés les transforme avec un rendement pratiquement quantitatif en acides α-chlorés correspondants. Cette réaction est en outre accompagnée d'un dégagement quantitatif de N_2O, ce qui permet d'ailleurs le dosage de l'azote aminé[11-15]:

$$\text{Alk—CHNH}_2\text{—COOH} + \text{HCl} + \text{HNO}_3 \rightarrow \text{Alk—CHCl—COOH} + \text{H}_2\text{O} + \text{N}_2\text{O}$$

Ces acides α-chlorés étant volatils, on pouvait penser à appliquer cette réaction au problème ici envisagé. Cependant la séparation des acides se heurtant en chromatographie gazeuse à des difficultés non négligeables, il nous a semblé préférable de poursuivre la transformation des acides en leur esters méthyliques par action du diazométhane:

$$\text{Alk—CHCl—COOH} + \text{CH}_2\text{N}_2 \rightarrow \text{Alk—CHCl—COOCH}_3 + \text{N}_2$$

Le problème de l'analyse d'un mélange d'acides aminés est ainsi ramené à celui du mélange des esters méthyliques chlorés correspondants. L'expérience montre alors qu'il est aisé par chromatographie gazeuse d'obtenir une excellente séparation des esters méthyliques des acides chlorés correspondant à glycocolle, alanine, acide α-aminobutyrique, valine, norvaline, leucine, isoleucine et norleucine. Les temps de rétention sur colonne huile de silicone–acide stéarique et pour les conditions données dans la partie expérimentale sont donnés dans le Tableau I. Un mélange de ces huit acides aminés transformés en esters méthyliques chlorés fournit ainsi le chromatogramme de la Fig. 1. La séparation obtenue est absolument remarquable et fournit une méthode possible de recherche de la norvaline et de la norleucine ainsi qu'une distinction particulièrement aisée entre leucine et isoleucine.

TABLEAU I

Ester méthylique correspondant à	*Temps de rétention*
Glycocolle	3′ 30″
Alanine	4′ 00″
Acide α-aminobutyrique	6′ 47″
Valine	9′ 53″
Norvaline	11′ 46″
Leucine	15′ 35″
Isoleucine	18′ 00″
Norleucine	20′ 50″

Si l'on aborde le problème pour un hydrolysat protéique, il faut s'assurer que les autres acides aminés neutres habituellement rencontrés ne viendront pas produire d'interférences. (Les acides aminés basiques et acides pourraient en effet, si nécessaire, être facilement éliminés.) Ceci limite en pratique le problème à la phénylalanine, sérine, thréonine, cystine et méthionine. Traitées dans les mêmes conditions, la phénylalanine ne fournit pas de pic; la sérine dont nous disposions fournit deux pics dont l'un très voisin de celui de la leucine; la thréonine, un pic qui interfère avec l'isoleucine; la cystine deux pics dont l'un voisin de celui du glycocolle; la méthionine rien.

Ces interférences sont gênantes surtout si l'on veut aborder le point de vue quantitatif, aussi avons nous étudié un autre remplissage de colonne soit le polyéthylèneglycol. Avec ce dernier et dans les conditions décrites dans la partie expérimentale, on obtient les temps de rétention du Tableau II. Un mélange des 8 acides aminés transformés en esters méthyliques chlorés fournit alors la Fig. 2. On n'observe plus que 7 pics, le glycocolle et l'acide α-aminobutyrique sortant ensemble, mais la phénylalanine, sérine, thréonine, méthionine ne donnent aucun pic gênant, seule la cystine donne deux pics, faibles d'ailleurs, l'un, en avant du glycocolle l'autre aux environs de la norvaline.

TABLEAU II

Ester méthylique correspondant à	*Temps de rétention*
Glycocolle	7′ 16″
Alanine	5′ 19″
Acide α-aminobutyrique	7′ 16″
Valine	8′ 46″
Norvaline	10′ 24″
Leucine	11′ 24″
Isoleucine	13′ 45″
Norleucine	15′ 40″

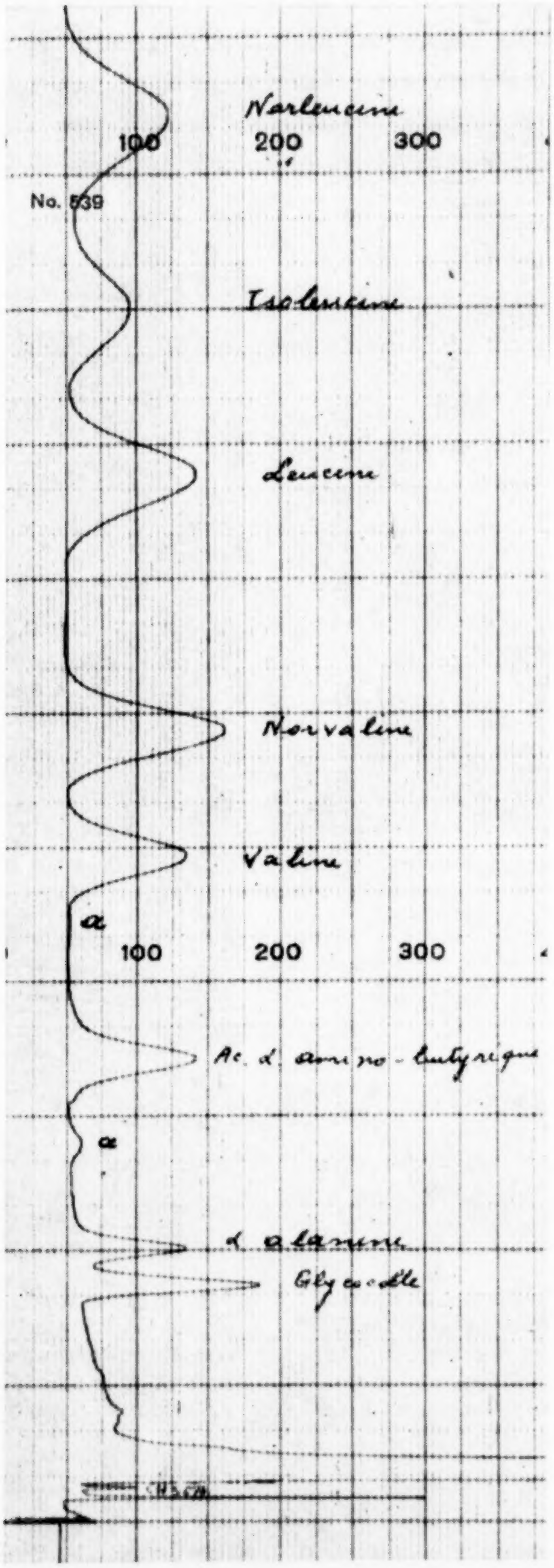

Fig. 1. Séparation, sur colonne huile de silicone–acide stéarique, des esters méthyliques chlorés correspondant à glycocolle, alanine, acide α-aminobutyrique, valine, norvaline, leucine, isoleucine, norleucine. a. Pics correspondant à des artéfacts.

Le mieux est alors d'utiliser les deux colonnes l'une à la suite de l'autre ce qui fournit le chromatogramme ci-dessous ou de nouveau les 8 acides aminés sont parfaitement séparés (Fig. 3).

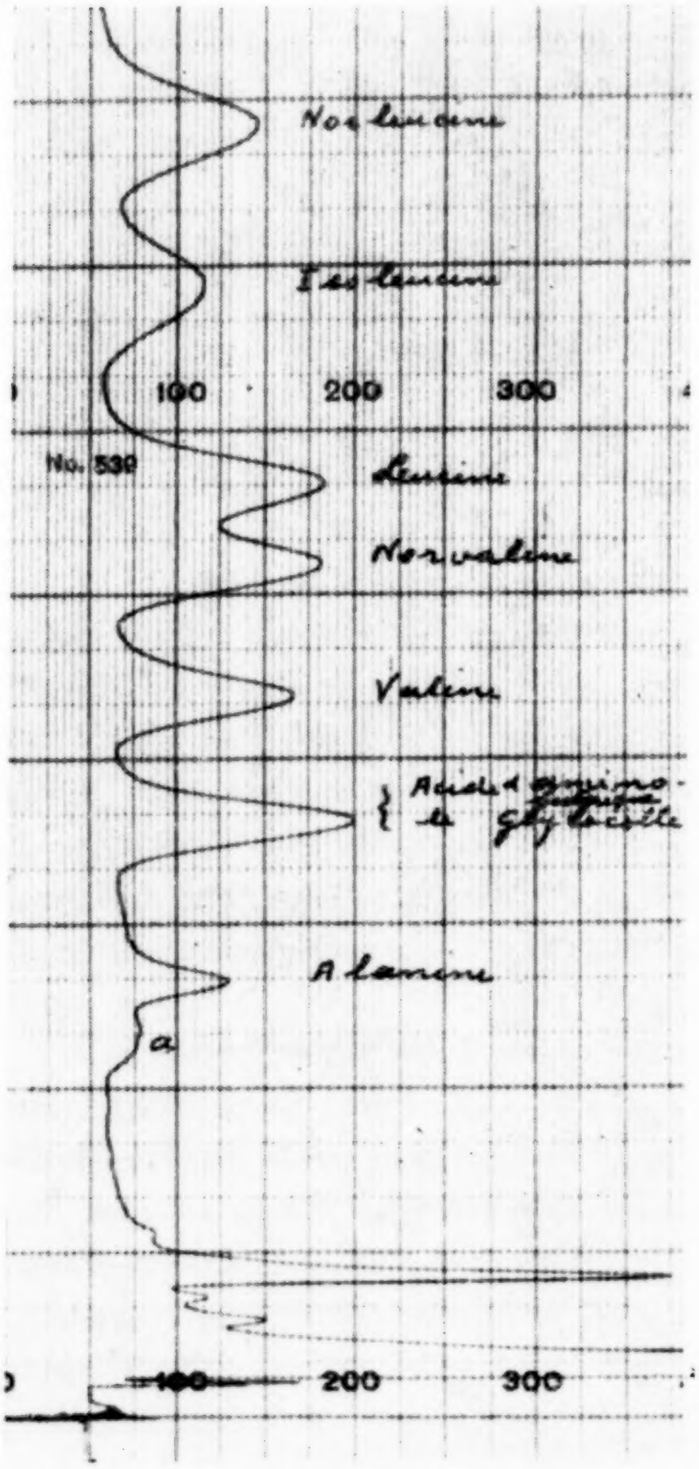

Fig. 2. Séparation, sur colonne polyéthylèneglycol, des esters méthyliques chlorés correspondant à glycocolle, alanine, acide α-aminobutyrique, valine, norvaline, leucine, isoleucine, norleucine. a. Pic correspondant à un artéfact.

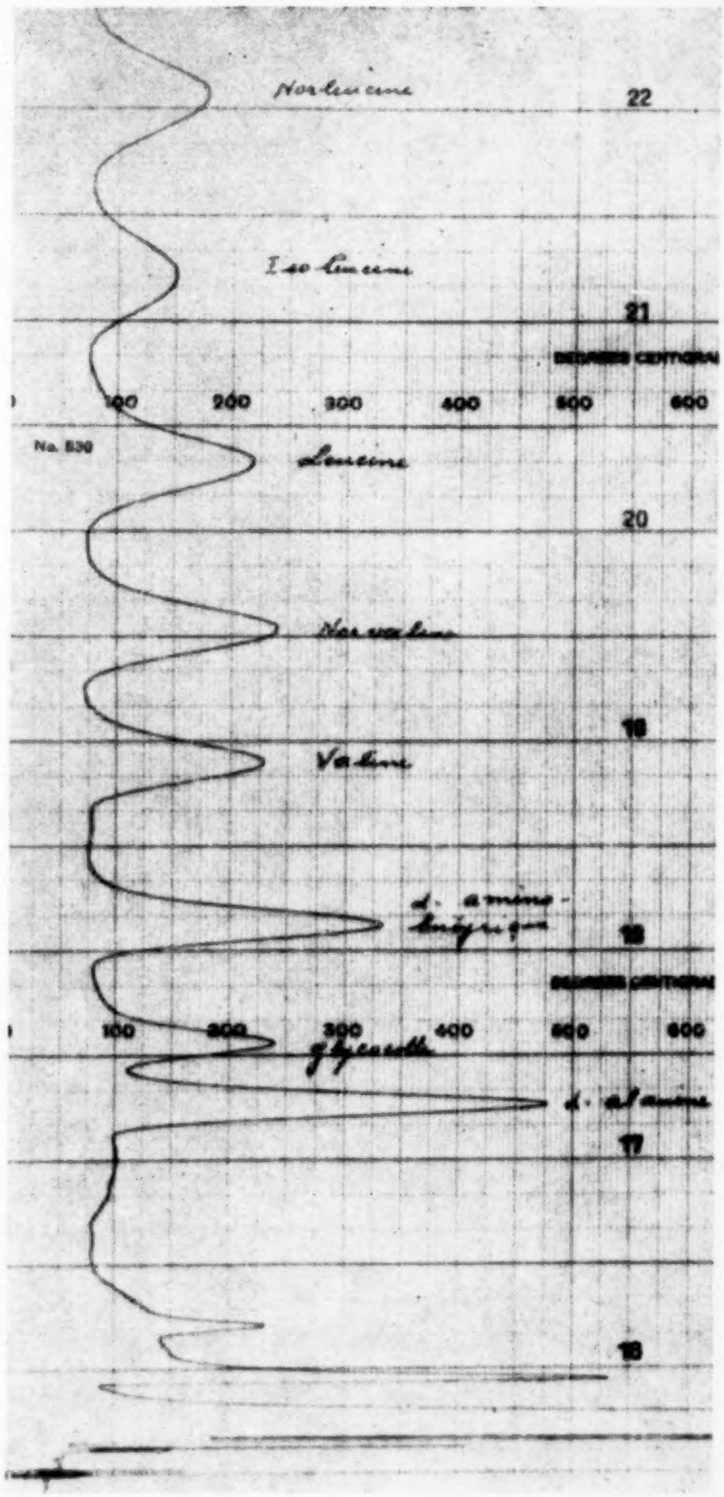

Fig. 3. Séparation, sur colonne polyéthylèneglycol puis huile de silicone–acide stéarique, des esters méthyliques chlorés correspondant à glycocolle, alanine, acide α-aminobutyrique, valine, norvaline, leucine, isoleucine, norleucine.

ESSAIS QUANTITATIFS

Un hydrolysat de caséine traité comme le mélange synthétique d'acide aminés fournit le chromatogramme de la Fig. 4. Nous sommes, d'autre part, parti d'un mélange de glycocolle, alanine, valine, leucine et isoleucine dans les proportions correspondant à la composition de la caséine. Ce mélange traité comme les précédents, donne la

Fig. 5. La bonne concordance entre les chromatogrammes des Figs. 4 et 5 permet de penser que la méthode pourra servir au dosage des acides aminés neutres. Ajoutons que les chromatogrammes obtenus à partir d'hydrolysats protéiques montrent qu'il n'est pas nécessaire d'éliminer les acides aminés basiques ou acides.

Pour l'instant, nous avons l'intention d'utiliser surtout la méthode à une recherche systématique de la présence (ou de l'absence) de norvaline ou de norleucine dans les produits naturels.

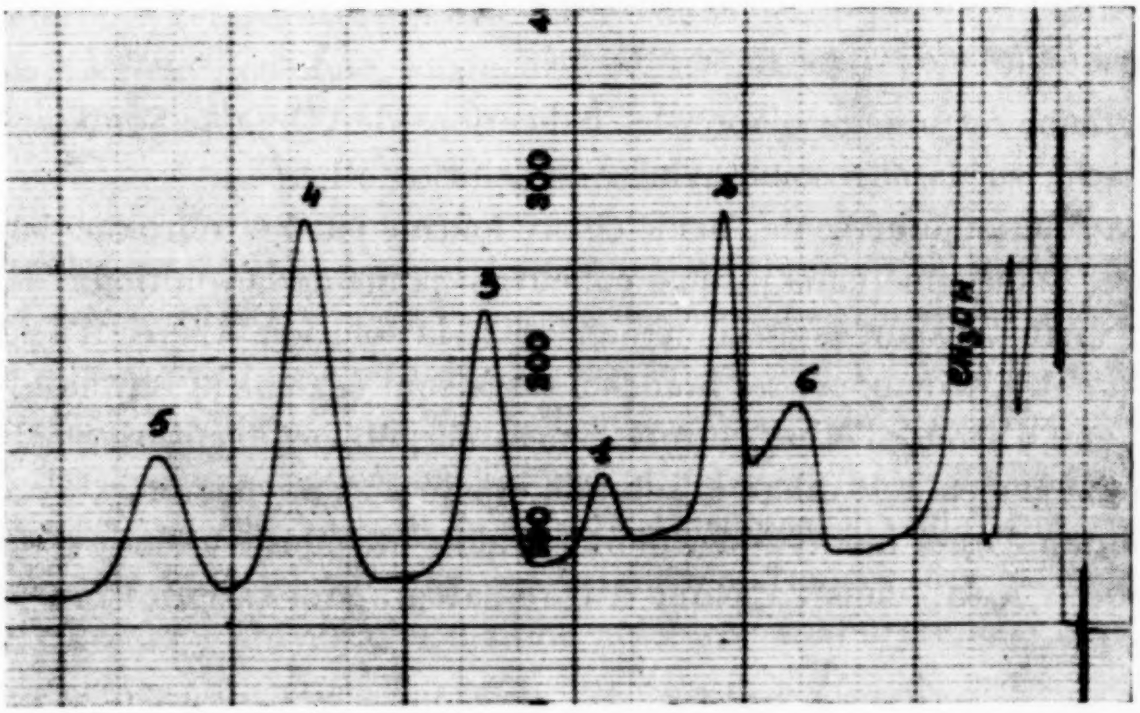

Fig. 4. Chromatogramme donné sur colonne polyéthylèneglycol par un hydrolysat de caséine. (1) glycocolle, (2) alanine, (3) valine, (4) leucine, (5) isoleucine, (6) probablement H_2O.

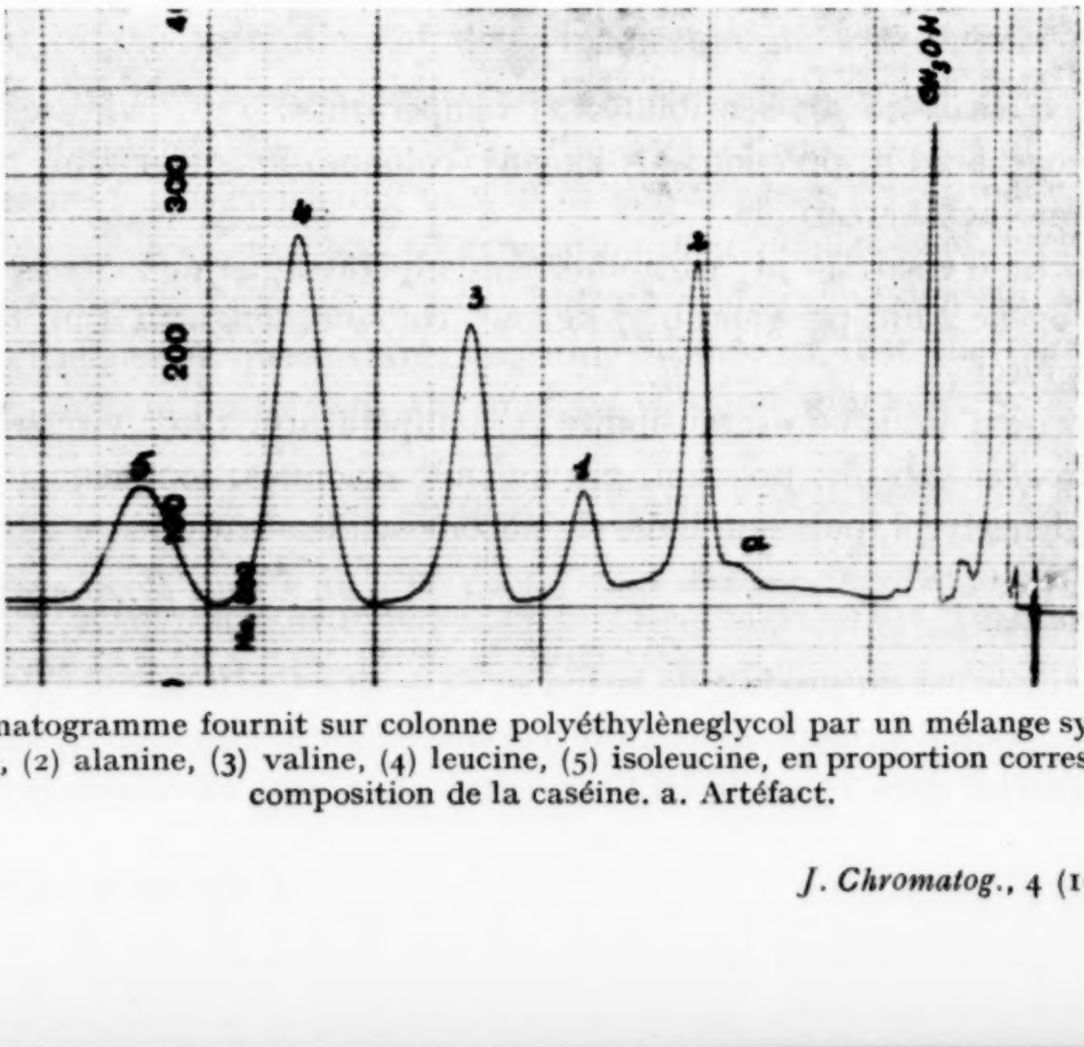

Fig. 5. Chromatogramme fournit sur colonne polyéthylèneglycol par un mélange synthétique de (1) glycocolle, (2) alanine, (3) valine, (4) leucine, (5) isoleucine, en proportion correspondant à la composition de la caséine. a. Artéfact.

PARTIE EXPÉRIMENTALE

(1) *Obtention d'un acide chloré à partir d'un acide aminé*

On ajoute à l'acide aminé un nombre de ml de HCl concentré numériquement égal à 3 fois le poids exprimé en gramme de cet acide aminé et la quantité calculée de HNO_3 concentré nécessaire pour former le nitrate de l'acide aminé majorée de 10%. On chauffe au bain-marie pendant 1 heure. On laisse refroidir et on extrait avec 1 ml d'éther. On centrifuge et on sépare la couche éthérée qui contient l'acide chloré.

(2) *Passage à l'ester méthylique correspondant*

Le diazométhane est obtenu d'après les indications de "*Organic Syntheses*"[16], action de KOH à 40% sur la nitrosométhylurée en solution éthérée.

Dans la solution éthérée de l'acide chloré fournie par l et refroidie dans la glace, on fait passer le diazométhane jusqu'à coloration jaune de la solution d'acide chloré. Le tube en verre amenant le diazométhane dans la solution éthérée d'acide chloré a la forme d'un M. Les deux petites branches du M sont également refroidies dans l'eau glacée, ceci afin d'arrêter l'éther du tube générateur, éther qui entraîné par le courant de diazométhane viendrait diluer la solution etheré d'acide chloré.

La solution d'ester méthylique chloré est alors introduite à l'aide d'une seringue micrométrique "Agla" dans l'appareil à chromatographie gazeuse (Fractovap de la firme Carlo Erba).

(3) *Chromatogrammes des Figs. 1, 2 et 3*

Pour l'obtention de ces chromatogrammes, les opérations décrites en (1) et (2) ont été conduites à partir d'un mélange de 100 mg environ de chacun des acides aminés suivants: glycocolle, alanine, acide α-aminobutyrique, valine, norvaline, leucine, isoleucine, norleucine.

Conditions opératoires chromatographiques

Fig. 1: prise d'essai: 40 μl; sensibilité: 2; température: 130°; vitesse du courant gazeux: hydrogène 2 l/h; pression: 0.5 kg/cm²; colonne: longueur 2 m; remplissage: huile de silicone–acide stéarique.

Fig. 2: prise d'essai: 40 μl; sensibilité: 2; température: 130°; vitesse du courant gazeux: hydrogène 2 l/h; pression: 0.37 kg/cm²; colonne: longueur 2 m; remplissage: polyéthylèneglycol.

Fig. 3: prise d'essai: 60 μl; sensibilité: 1; température: 130°; vitesse du courant gazeux: hydrogène 2.6 l/h; pression: 0.8 kg/cm²; colonnes: longueur 4 m, d'abord 2 m polyéthylèneglycol, puis 2 m huile de silicone–acide stéarique.

(4) *Essai quantitatif*

La caséine utilisée est un produit de la Fisher Scientific Co. (Caséine C.P.).

Dans un appareil à reflux, on chauffe à douce ébullition au bain de sable pendant 23 h, 5 g de caséine dans 15 ml de HCl concentré. On ajoute alors avec précaution 4 g de norite et on continue l'ébullition encore pendant 1 h. On laisse refroidir et on filtre. La solution obtenue est saturée par un courant de HCl gazeux et on répète sur 2 ml de cette solution les opérations (1) et (2) précédentes. La solution éthérée des esters chlorés obtenus donne la Fig. 4.

Le chromatogramme de la Fig. 5 est obtenu à partir d'un mélange initial synthétique de: glycocolle 20 mg, alanine 32 mg, valine 72 mg, leucine 92 mg, isoleucine 61 mg, que l'on traite suivant (1) et (2).

Les chromatogrammes des Figs. 4 et 5 sont pris dans les mêmes conditions soit: sensibilité: 2; température: 130°; vitesse du courant gazeux: hydrogène 2.5 l/h; pression: 0.35 kg/cm^2; colonne: longueur 2 m; remplissage: polyéthylèneglycol.

RÉSUMÉ

Le mélange HCl + HNO_3 concentrés permet de transformer avec un rendement pratiquement quantitatif les acides α-monoaminés monocarboxylés en acides α-chlorés correspondants. Cette réaction a été appliquée, moyennant la transformation ultérieure des acides α-chlorés en esters méthyliques par action du diazométhane, à la séparation des acides α-monoaminés monocarboxylés par chromatographie gazeuse. Une colonne de 4 mètres, 2 mètres de polyéthylèneglycol suivi de 2 mètres de huile de silicon–acide stéarique fournit une séparation remarquable des esters méthyliques chlorés correspondant à glycocolle, alanine, acide α-aminobutyrique, valine, norvaline, leucine, isoleucine, norleucine. Un essai quantitatif permet de penser que le dosage de ces acides aminés dans les protéines pourra se faire facilement par ce procédé.

SUMMARY

By treating α-monoamino-monocarboxylic acids with a mixture of conc. HCl + HNO_3, the corresponding α-chloro-acids are obtained in a yield of almost 100%. This reaction was applied to the separation of the α-monoamino-monocarboxylic acids by gas chromatography, the chloro-acids having first been converted to their methyl esters by means of diazomethane.

A column of 4 m consisting of 2 m of polyethylene glycol followed by 2 m of silicon oil–stearic acid gave a good separation of the chloro-acid methyl esters corresponding to glycine, alanine, α-aminobutyric acid, valine, norvaline, leucine, isoleucine, norleucine. A quantitative experiment showed that the determination of these amino-acids in proteins by this method will probably be easy.

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Short Communications

Fractionation of polyphenolic substances from cacao beans by paper electrophoresis

Cacao beans must be subjected to fermentation before they are used in chocolate making. The most striking changes during fermentation are those that occur in the polyphenolic substances in the cotyledons. The nature and extent of these transformations are still a matter of investigation, and there is a definite need for a method of separating and identifying the polyphenols from fermented and unfermented beans.

Previous work was mainly carried out by paper chromatography of HCl extracts[1-3].

Phenolic compounds[4,5] and also leucocyanidins[6] have been separated by paper electrophoresis.

This paper describes a procedure for fractionating polyphenols from cacao beans by paper electrophoresis.

Experimental

Material and methods. Beans of the Forastero type, from Bahia, were used throughout. For the polyphenolic extracts, 100 g of freshly dried cotyledons were blended in a Waring blendor with 200 ml of chilled 0.1 N HCl at 0°, three times for five minutes each, and then filtered. For electrophoresis, 50 μl were placed in a band of 3.8 cm on strips (4.8 × 40 cm) of unwashed Whatman No. 1 paper.

Apparatus and procedure. Electrophoresis was carried out in a KELAB apparatus, the following conditions giving the best results: borate buffer of pH 10 as electrolyte, 200 V/cm, 6 hours. The polyphenolic fractions were detected by immersing the strips in $Fe{-}Cl_3{-}K_3Fe(CN)_6$ reagent as, described by BARTON[7]. *et al.* For quantitative estimation by densitometry, it is better to use a more dilute reagent (1:3 in distilled water), and then wash the strips in 0.1 N HCl and in distilled water before air drying. The fractions were visible as blue bands.

Chromatographic resolution of the fractions. Each fraction was cut off from strips that had been submitted to electrophoresis at the same time, but had not been treated with the BARTON reagent, and eluted with 70% methanol–1.0 N HCl (95:5 v/v). Elution by capillary methods is very time-consuming. If the sections were soaked and stirred with a definite amount of eluant in a centrifuge tube, a clear supernatant could be obtained by centrifuging the mixture for 10 min at 3000 r.p.m. Each sample was concentrated in vacuo at 45° and chromatographed. For one-dimensonal chromatograms, n-butanol–formic acid–water (10:2:5 v/v) was used at 25° for 18 h; for two-dimensional chromatograms the first solvent was distilled water, according to ROBERTS AND WOOD[8], and the second the above-mentioned butanol–formic acid–water mixture. The respective polyphenolic components were isolated from the spots of each fraction, and identified by specific reactions and spectrophotometry.

Results and discussion

Figs. 1 and 2 represent typical electropherograms, of unfermented and fermented beans respectively. Each of these patterns was reproducible in all experiments, with remarkable constancy. On the electropherograms of extracts of unfermented beans five fractions are visible. The first (I) is a complex tannic material, which does not move; fraction II gave a pink colour with vanilline–HCl reagent[3] like the leucocyanidins. Fraction III was identified as anthocyanidin glycosides by specific reactions[9] and spectrophotometry[10].

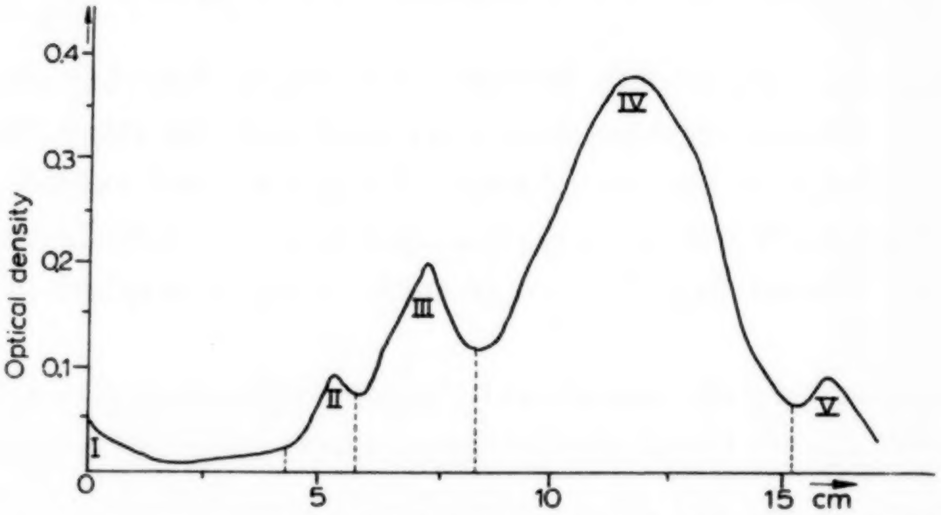

Fig. 1. Electropherogram of unfermented cacao beans. Conditions: borate buffer of pH 10: 200 V/cm; 6 h.

Fraction IV proved to be of phenolic nature, giving a characteristic reaction with *p*-nitroaniline[11] and toluene-*p*-sulphonic acid[12]. In addition, a non-identified fraction is present, which also shows phenolic character. The number and the nature of the components isolated from each fraction, as well as a discussion about the biochemistry of cacao fermentation will be the subject of a further paper. As can be seen in Fig. 2,

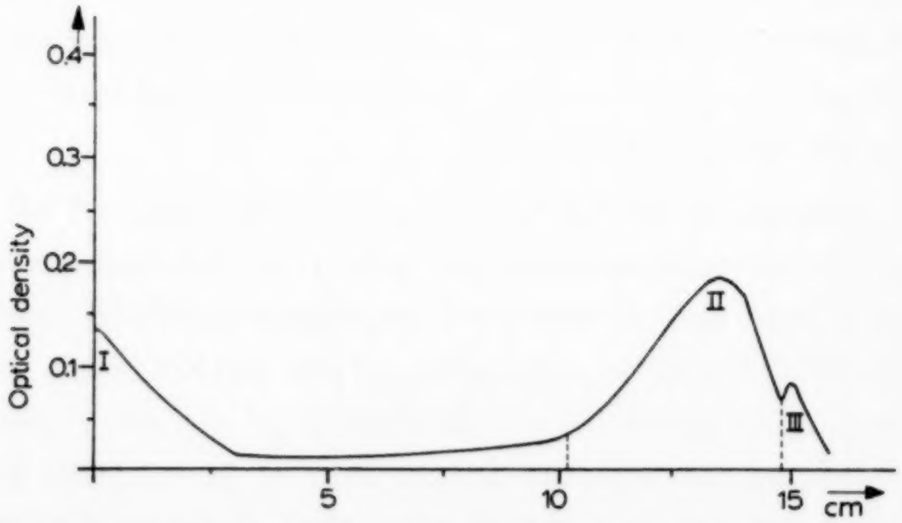

Fig. 2. Electropherogram of fully fermented cacao beans. Conditions: borate buffer of pH 10; 200 V/cm; 6 h.

the non-mobile fraction is greatly increased in fermented beans; it consists of condensed tannins. The anthocyanin fraction failed to appear, which agrees with former experiments[13,14] and proves that the anthocyanins are hydrolysed during fermentations.

Fraction III decreased in fermented beans; this is in accordance with the already observed loss of catechins during fermentation, which causes the characteristic browning of fermented beans.

In an attempt to correlate the degree of fermentation with the electrophoretic behaviour, beans were tested that had been rejected from commercial batches because they had been labeled "insufficiently fermented" judged by a standardized method[15]. Intermediary patterns were always found, as in Fig. 3.

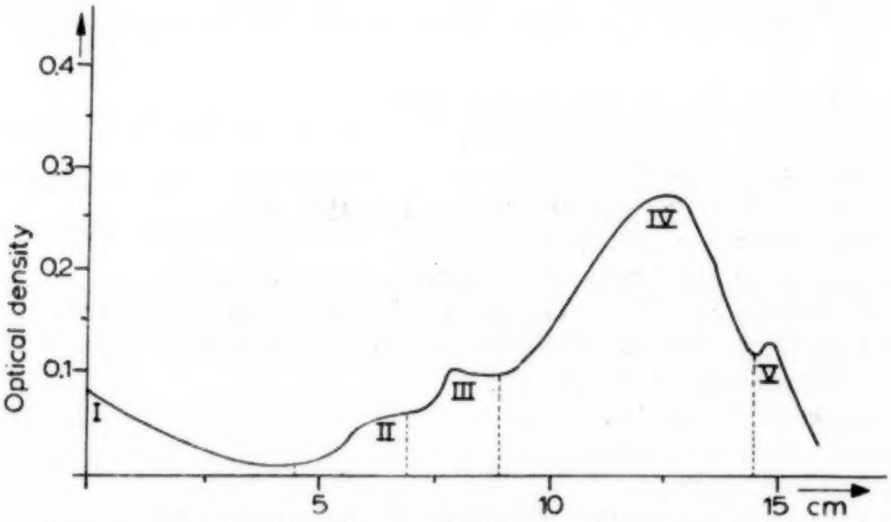

Fig. 3. Electropherogram of under-fermented cacao beans. Conditions: borate buffer of pH 10; 200 V/cm; 6 h.

The fraction corresponding to the anthocyanins appears to be incompletely transformed. It has already been proved that the transformation of the polyphenols during the fermentation procedure is a progressive process[14,15].

Advantages of the method

The greatest advantages are speed, sensitivity and constancy of results. Since the commercial value of the beans is related to the degree of fermentation, this procedure may find practical application in determining this degree.

Limitations and precautions to be taken

(a) Great care must be exercised because of the powerful polyphenoloxidase system present in the cotyledons; although FORSYTH[2] mentioned that in powdered preparations the polyphenolic components remained unchanged, we have always obtained unsatisfactory results with such preparations.

(b) The electrophoresis should be carried out as soon as possible after the extraction of the polyphenols, in view of their instability in acid solution.

(c) By drying the strips at 50° for ten minutes, after the electrophoretic migration has taken place, better definition can be obtained.

Conclusions

1. The polyphenolic components of cacao beans can be fractionated by paper electrophoresis.
2. Unfermented and fermented beans both give typical electropherograms.
3. The procedure described provides a means of determining the degree of fermentation of the beans.

Acknowledgements

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J. Chromatog., 4 (1960) 347–350

Diazotised *p*-nitroaniline as a location reagent for ammonium salts of aliphatic acids

The most used reagents for location of ammonium salts of aliphatic acids after chromatography have been acid–base indicators, of which a variety have been recommended by various workers. These reagents suffer from the disadvantages of:

(i) Difficulty in distinguishing between true spots and artefacts.
(ii) Instability of the colours produced.
(iii) Necessity of removing the solvent completely before spraying.

Nesslers reagent has also been suggested as a location reagent but suffers from disadvantages (ii) and (iii) above, and also that the high alkali concentration renders the paper difficult to handle. Diazotised *p*-nitroaniline may be used as a spray reagent to overcome these difficulties. This reagent is used in inorganic analysis as a spot test for ammonium ion, but its use in chromatography has been limited to the location of phenolic compounds.

J. Chromatog., 4 (1960) 350–351

The reagent was prepared as follows: Dissolve *p*-nitroaniline (1 g) by warming in a mixture of concentrated hydrochloric acid (2 ml) and water (20 ml), and dilute to 180 ml. After cooling to room temperature add 5 % (w/v) sodium nitrite (20 ml) and shake until all the solid has dissolved. Stored at 4° the reagent can still be used after 3 months, any deposit being filtered off before use.

After development in an ammoniacal solvent, sheets are removed from the tank and hung in a fume-free atmosphere at room temperature for about 15 minutes. While still just damp, sheets are sprayed lightly and evenly with the diazotised *p*-nitroaniline reagent and then immediately resprayed with 15 % (w/v) aqueous sodium carbonate, when ammonium compounds appear as red spots. The papers are then hung to dry at room temperature in a fume-free atmosphere. The background colour may be high at first, but will clear as the paper dries, eventually assuming a pale yellow-brown colour. The red colours are stable for several weeks if the paper is kept in a fume-free atmosphere.

The sensitivity of the reagent is such that 0.1 μmole of the ammonium salts of monobasic acids was readily detected. Good definition has been obtained with a wide variety of compounds including the following, $C_1 - C_9$ fatty acids, α-hydroxyisocaproic, α-hydroxy-isovaleric, α-keto-glutaric, α-keto-isocaproic, malic, lactic, succinic, citric, tartaric, glycollic, tiglic, dimethacrylic and methacrylic acids.

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Addendum

(to publication entitled: RAPID PAPER IONOPHORESIS USING ORGANIC BUFFERS IN WATER–FORMAMIDE AND WATER–UREA, by L. N. WERUM, H. T. GORDON AND W. THORNBURG, *J. Chromatog.*, 3 (1960) 125–145)

The values of "ionic strength" stated for the various buffers described in this paper were calculated as the sum of the effective molar concentration of all ions. For each ion, the effective molar concentration is its actual molarity multiplied by the square of its valence. By this definition, the "ionic strength" of a 0.1 *M* solution of sodium chloride would be 0.2, and of calcium chloride, 0.6.

The conventional definition of ionic strength, first given by G. N. LEWIS, is *one-half* of the sum of the effective molar concentrations of all the ions. To avoid errors in comparison of ionic strengths, values in this paper should be converted to LEWIS ionic strength by dividing by 2.

Received May 2nd, 1960

BOOK REVIEW

Chromatographie en chimie organique et biologique, Vol. II, publié sous la direction de E. LEDERER, Masson et Cie Éditeurs, Paris, 1960, xiv + 876 pages.

Volume II of this treatise on chromatography (for the review of Vol. I see *J. Chromatog.*, 3 (1960) 507) contains the following chapters:

I. Sugars, by R. DEDONDER, 71 pages
II. Phosphate esters, by J. MONTREUIL, 28 pages
III. Sulphur compounds, by P. FROMAGEOT, 60 pages
IV. Sterols, steroids and related compounds, by R. NEHER, 85 pages (this is an abbreviated version of the review which appeared in *J. Chromatog.*, 1 (1958) 122, 205)
V. Lipids, by J. ASSELINEAU, 25 pages
VI. Amino-alcohols, by M. JUTISZ, 17 pages
VII. Amino-acids and peptides, by M. JUTISZ, 102 pages
VIII. Derivatives of amino-acids, by R. MONIER, 23 pages
IX. Proteins, by M. JUTISZ, 46 pages
X. Purines, pyrimidines and derivatives, by P. BOULANGER AND J. MONTREUIL, 102 pages
XI. Pigments (non-nitrogenous), by E. LEDERER, 10 pages
XII. Pigments (nitrogenous), by G. BISERTE, 54 pages
XIII. Fat-soluble vitamins, by E. LEDERER, 9 pages
XIV. Water-soluble vitamins and growth factors, by R. MUNIER, 108 pages
XV. Nitrogenous hormones, by J. ROCHE, R. MICHEL AND J. NUNEZ, 26 pages
XVI. Antibiotics, by E. SAIAS, 28 pages.

Alphabetic reference lists follow each chapter and there is an author and a subject index at the end of the volume.

As stated in the preface one aim of the book, namely to present a text permitting execution of chromatograms without reference to the original papers, was only partly achieved. There are, however, numerous methods for the preparation of adsorbents, columns etc. in the text, usually in small print. The book is extremely well produced and will prove invaluable to all French-speaking biochemists and chemists.

J. Chromatog., 4 (1960) 352

NEW BOOKS

Papierelektrophorese: Grundlagen–Methodik–Klinik, by ANNO DITTMER 2nd Ed , VEB Gustav Fischer Verlag, Jena, 1960, 272 pages, price DM 22.–.

Handbuch der Papierchromatographie, Band II. *Bibliographie und Anwendungen*, by I. M. HAIS AND K. MACEK, VEB Gustav Fischer Verlag, Jena, 1960, xxiv + 726 pages, price DM 44.–.

A translation of the second Czech edition by J. LIEBSTER.

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THE INFLUENCE OF WATER AND ACID CONCENTRATION ON THE CHROMATOGRAPHIC BEHAVIOUR OF INORGANIC IONS

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(Received February 11th, 1960)

In a previous paper[1] we showed that the shape of the curves of R_F *vs.* acid concentration of the eluant was almost the same ("bell curve"). Since in that paper no systematic variations of $[H_2O]$ and [HCl] were made, it was not possible to determine to what extent each one of the components influenced the R_F. The present work was carried out in order to obtain a better interpretation of the results obtained and, at the same time, to determine better conditions for separation.

Whilst this work was in progress, we read a paper by ALMÁSSY AND DESZÖ[2], which in part was similar to our work, but which was less extensive and contained only limited conclusions. The authors merely concluded that the length of the spots varies inversely with the water and acid concentration of the eluant. The results of the present work have confirmed these findings, although this was not our main purpose.

EXPERIMENTAL

The ascending method, already described in a previous paper[1], was used throughout this work.

The eluants used were mixtures of $HCl + H_2O + n$-butanol, with only one phase, in order to avoid having to determine the acid and water in the organic phase, which is necessary when two phases are obtained.

Eight series of five eluants each were prepared in such a way that in the first four series the [HCl] was maintained at four different levels and the $[H_2O]$ was systematically varied, while in the last four series $[H_2O]$ was fixed at four different levels and [HCl] was systematically varied.

The compositions of the eluants are summarized in Table I.

DISCUSSION

In order not to make this paper too extensive, the results are presented partly in graphs, partly in tables. However, as all the graphs were drawn, the comments on the work dealing with the elements whose R_F and R_M are tabulated, also refer to the curves obtained when these values are graphically represented.

TABLE I

COMPOSITION OF ELUANTS

Series A				*Series B*			
Designation	*[HCl] (g/100 ml)*	*[H_2O] (g/100 ml)*	*n-Butanol*	*Designation*	*[H_2O] (g/100 ml)*	*[HCl] (g/100 ml)*	*n-Butanol*
I.1	17.6	30.0	to 100 ml	I.1	60.0	37.2	to 100 ml
I.2	17.6	38.0	,,	I.2	60.0	29.0	,,
I.3	17.6	50.0	,,	I.3	60.0	21.9	,,
I.4	17.6	70.0	,,	I.4	60.0	15.4	,,
				I.5	60.0	9.6	,,
II.1	11.0	18.8	,,	II.1	40.0	24.9	,,
II.2	11.0	23.9	,,	II.2	40.0	19.3	,,
II.3	11.0	31.1	,,	II.3	40.0	14.6	,,
II.4	11.0	43.8	,,	II.4	40.0	10.3	,,
II.5	11.0	68.9	,,	II.5	40.0	6.4	,,
III.1	4.4	7.5	,,	III.1	20.0	12.4	,,
III.2	4.4	9.5	,,	III.2	20.0	9.7	,,
III.3	4.4	12.5	,,	III.3	20.0	7.3	,,
III.4	4.4	17.5	,,	III.4	20.0	5.1	
III.5	4.4	27.5	,,	III.5	20.0	3.2	,,
IV.1	0.9	1.5	,,	IV.1	5.0	2.9	,,
IV.2	0.9	1.9	,,	IV.2	5.0	2.3	,,
IV.3	0.9	2.5	,,	IV.3	5.0	1.8	,,
IV.4	0.9	3.5	,,	IV.4	5.0	1.3	,,
IV.5	0.9	5.5	,,	IV.5	5.0	0.8	,,

Some groups of elements are represented by two graphs, relating to series A and B of Table I. Two more graphs for each group of elements were drawn relating R_M with pH_2O and R_M with pCl^- respectively. These two graphs are necessary in order to apply the theoretical considerations developed by LEDERER in two papers[3,4], to the present work. In one of these papers[3], LEDERER AND KERTES established the following equation referring to an ion developed with an acid eluant on ion-exchange papers:

$$x\,\mathrm{pH} = R_M + \mathrm{const.} \tag{1}$$

in which x is the number of charges of the ion and $R_M = \log(1/R_F - 1)$, the term introduced by BATE-SMITH AND WESTALL[5].

The straight lines represented by equation (1) must be parallel in the case of ions of the same charge, a fact that was observed with several ions.

In the second paper[4] LEDERER developed these considerations further, and applied them to chloro-complexes of inorganic ions in partition chromatograms. The equilibrium of a complex formation

$$\mathrm{M(H_2O)}_x^{y+} + x\,\mathrm{Cl}^- \rightleftharpoons \mathrm{MCl}_x^{(y-x)} + x\,\mathrm{H_2O} \tag{2}$$

has the following constant:

$$K = \frac{[MCl_x]\cdot[H_2O]^x}{[M(H_2O)_x]\cdot[Cl^-]^x} \tag{3}$$

or

$$-\,pK = \log\left[\frac{MCl_x}{M(H_2O)_x}\right] + x\log[H_2O] + x\,pCl^- \tag{4}$$

Since

$$\log\left[\frac{MCl_x}{M(H_2O)_x}\right]$$

is the log of the partition coefficient, which varies directly with R_M, one may write:

$$-\,pK = R_M + x\log[H_2O] + x\,pCl^- + k \tag{5}$$

This equation would probably permit the determination of the "instability constant K" of the complex, if the partition coefficient (and therefore R_M) were only dependent on the complexation and not on the solubilities of the ion and the complex in the various eluants used.

In the present work when $[H_2O]$ was fixed, the following equation was applied:

$$-\,pK = R_M + x\,pCl^- + k'x + k \tag{6}$$

or

$$x\,pCl^- = -\,R_M - k'x - k - pK \tag{7}$$

Similarly, when [HCl] was fixed:

$$x\,pH_2O = R_M + k'x + k + pK \tag{8}$$

If the number of chloride ions x is constant, equations (7) and (8) are straight lines. If two ions give parallel lines for equations (7) or (8) with the same eluant, then they are probably of the same ionic species.

Group IA—rubidium, caesium

Notwithstanding the discontinuities in the [HCl] of the solutions represented by the curves in Fig. 1a, these curves show a smooth continuity, indicating that $[H_2O]$ is the ruling factor in the R_F values of these ions. The curves of Fig. 2a, showing four different and increasing levels of R_F for the increasing $[H_2O]$, confirm this assumption.

Variation of the complexation seems to be revealed by Fig. 2b.

The equilibrium equations may be more complex than equation (2):

$$M(H_2O)_x^{y+} + z\,Cl^- \rightleftharpoons M(H_2O)_xCl_z^{(y-z)} \tag{9}$$

or:

$$M(H_2O)_x^{y+} + zCl^- \rightleftharpoons M(H_2O)_{x'}Cl_z^{(y-z)} + (x - x')\,H_2O \tag{10}$$

The instability constants are:

$$K = \frac{[M(H_2O)_xCl_z]}{[M(H_2O)_x]}\cdot\frac{1}{[Cl^-]^z} \tag{11}$$

and

$$K = \frac{[M(H_2O)_{x'}Cl_z]}{[M(H_2O)_x]} \cdot \frac{[H_2O]^{(x-x')}}{[Cl^-]^z} \tag{12}$$

or

$$-\,pK = R_M + z\,pCl^- + k \tag{13}$$

and

$$-\,pK = R_M + z\,pCl^- - (x - x')pH_2O + k \tag{14}$$

Equation (14), referring to a complex hydrated ion, could explain the observed behaviour of the ions. In fact, even supposing that the ion remains with the same chloride complexation (*i.e.* z = constant), equation (14) will only be a straight line for pH_2O = constant if $(x - x')$ is also constant. But $(x - x')$ represents the number of moles of water separated from (or added to) the ion, which possibly varies with the acid concentration. Hence it would be easier to explain the non-linearity of the graphs of Fig. 2b by a variation in the hydration of the complex ion, than by the formation of new chloro-complexes, which are not described in the literature.

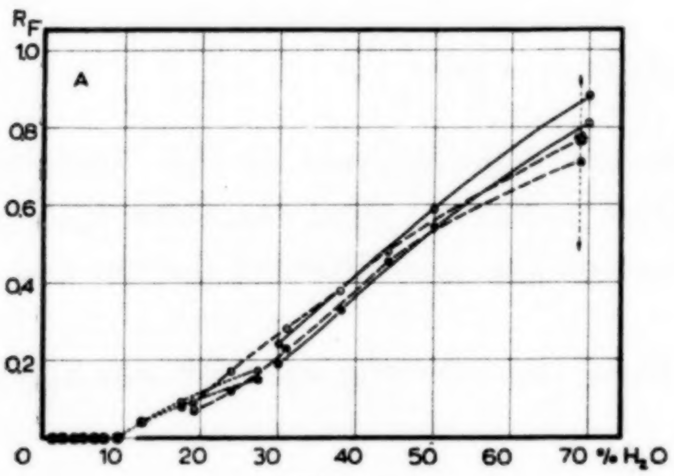


Fig. 1a

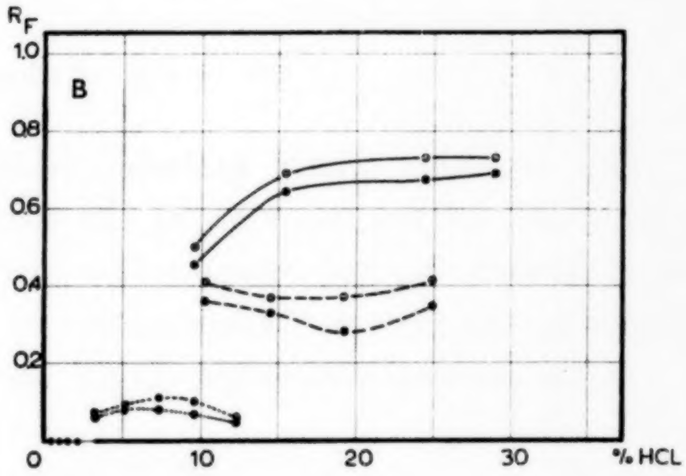


Fig. 2a

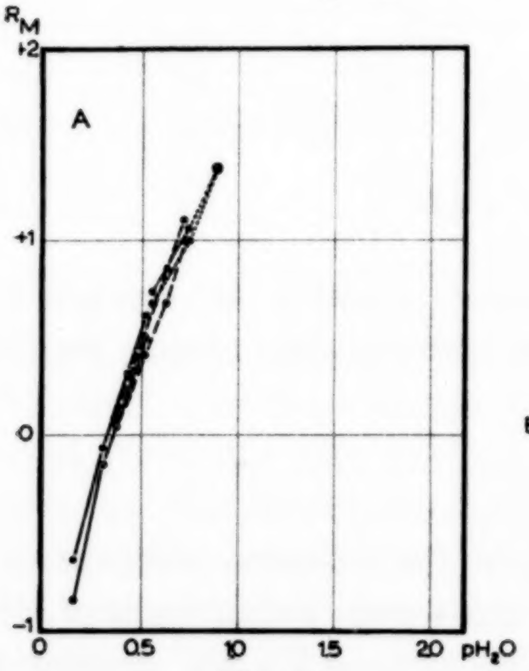
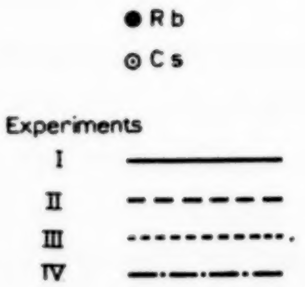


Fig. 1b



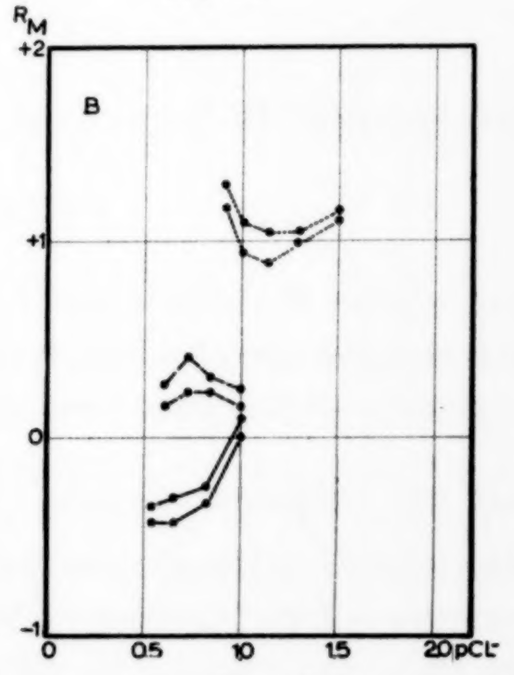


Fig. 2b

It must be pointed out that

(1) in this type of eluant the migration of ions starts only if the water content is higher than 10%;

(2) the best conditions for separation are those of Expt. B.II.2.

Group IB—copper, gold

The shape of the curves of Fig. 3a is quite different from that of Fig. 1a, since there is no continuity in the curves of each group of experiments. It can be seen that with increasing acid content the R_F value increases in the case of Cu and decreases in the case of Au, but by jumps, which can be interpreted as a chloro-complexation; the increase in the R_F would be due to the formation of a more anionic form and the decrease to a less anionic form of the complex.

In Fig. 3b several complexes can be noted in the Cu curves: one in Expt. A.I, two in Expt. A.II and three in Expt. A.III.

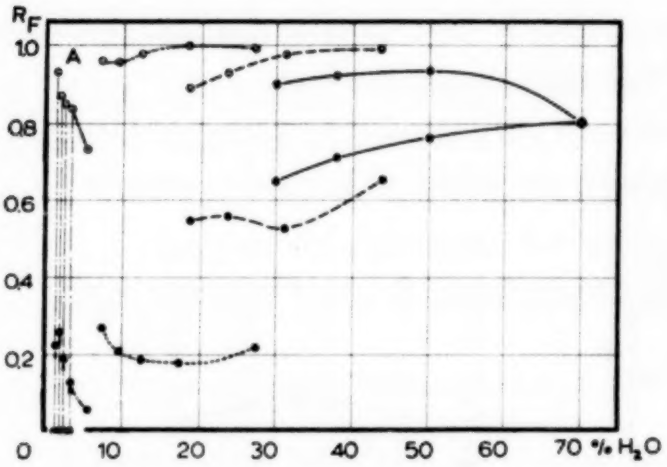


Fig. 3a

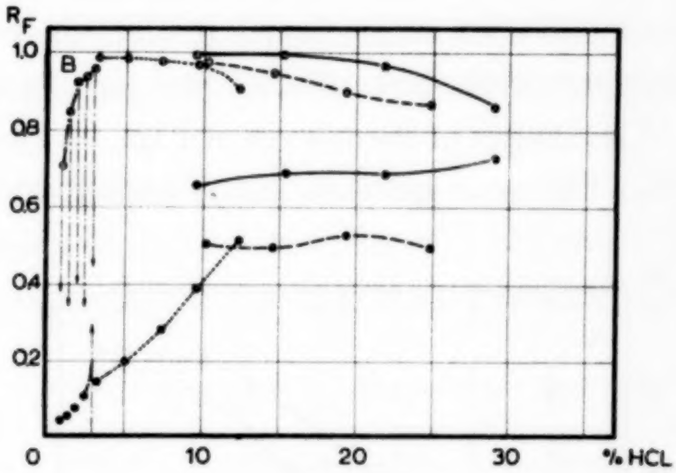


Fig. 4a

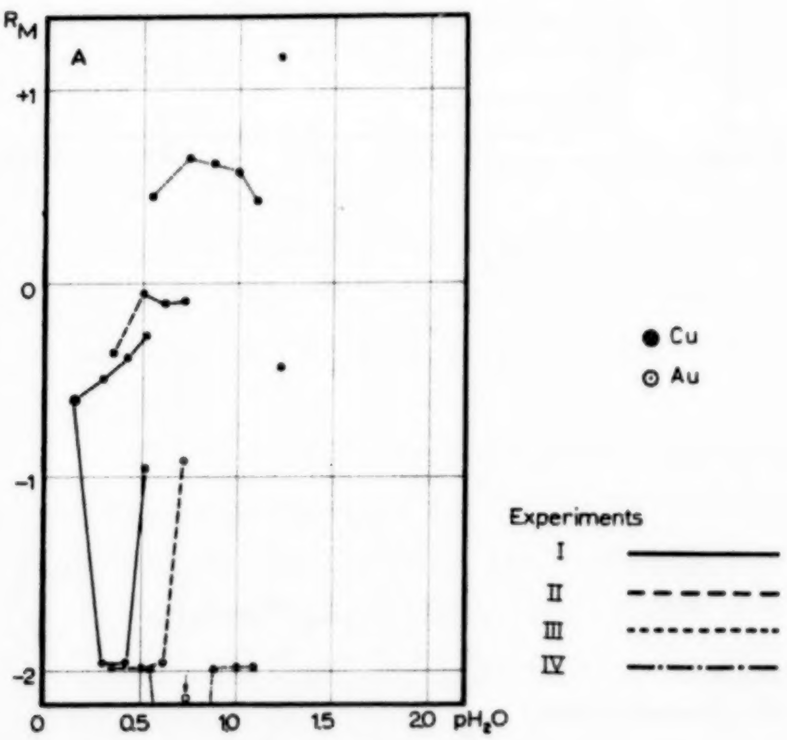


Fig. 3b

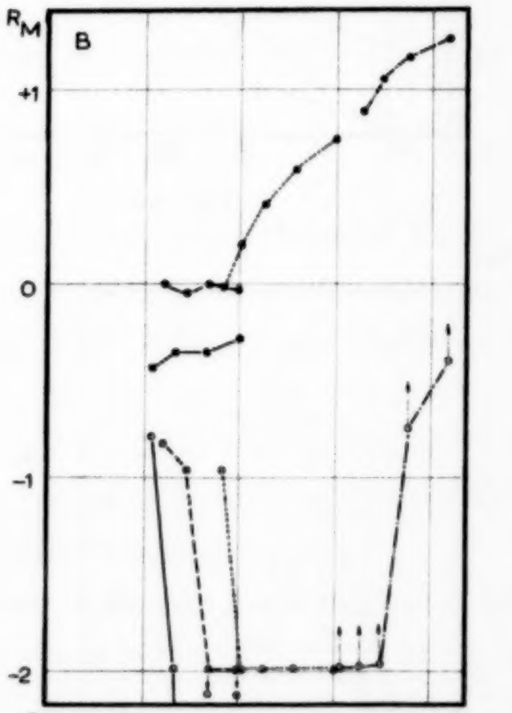


Fig. 4b

Au seems to have two different complexes in Expts. A.I and A.II, but as the R_F values are greater than 0.90, the corresponding R_M variations are rather difficult to interpret.

In Fig. 4a it can be seen that the influence of $[H_2O]$ on the R_F of Au is very small. In the case of Cu the [HCl] has no influence in Expts. B.I and B.II, but in Expts. B.III and B.IV there is a great increase of R_F as the [HCl] increases.

In Fig. 4b Expts. B.I show only one complex; Expts. B.II also show only one complex represented by a straight line parallel to the pCl^- axis and hence independent of $[Cl^-]$. Expts. B.III and B.IV probably represent only one ionic species.

With these eluants ion migration begins when the water content is higher than 1 %; the best conditions for separation of the two elements are those of Expts. A.III and B.III.

Group IIA—beryllium, magnesium

In this group, as in the alkali group, the $[H_2O]$ is the determining factor for the R_F values. However, in the 4.4 % HCl zone, the behaviour is different, as can be seen in Fig. 5a and even more clearly in Fig. 5b, where the Be line shows a distinct variation of angular coefficient, which must signify a different complexation.

The shapes of the curves of Figs. 6a and 6b are very similar to those of Group IA, so that the same considerations expounded there are applicable to this group.

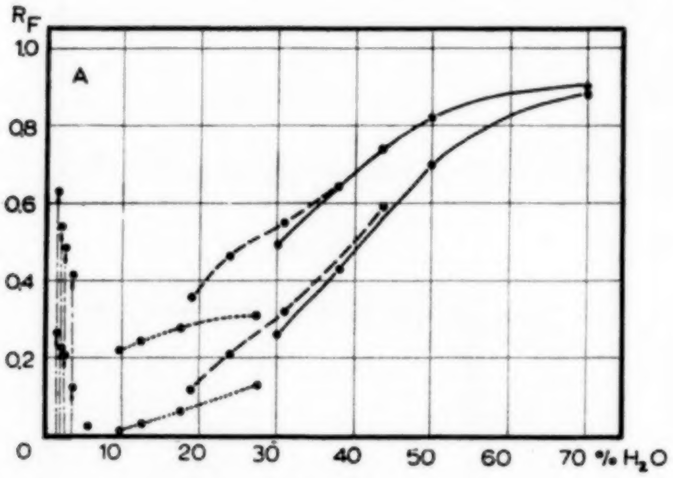


Fig. 5a

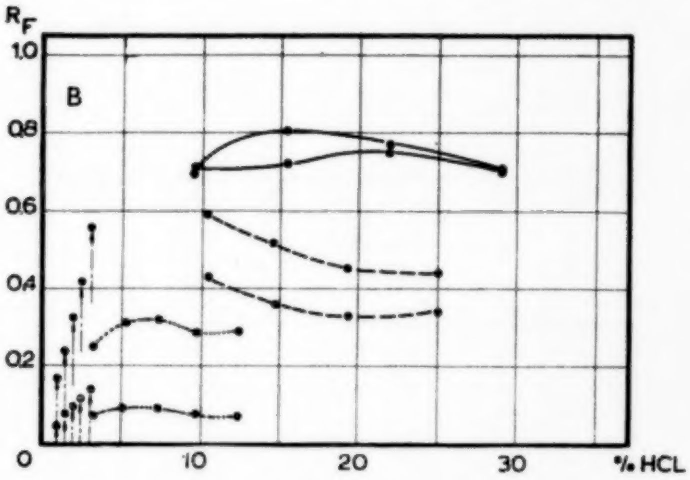


Fig. 6a

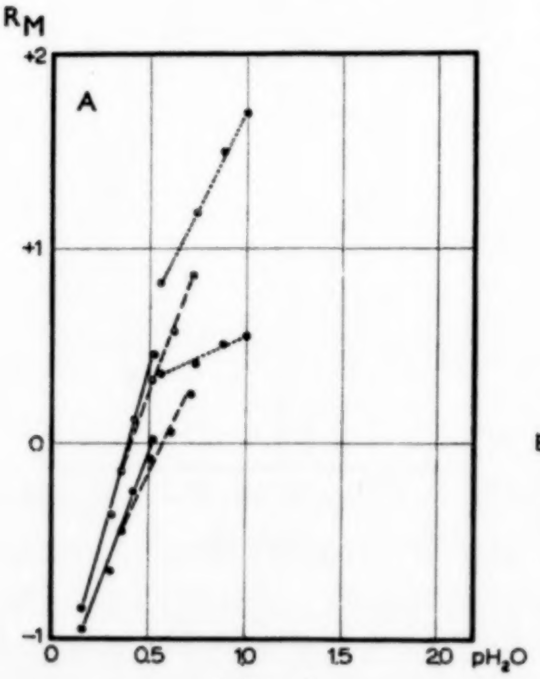
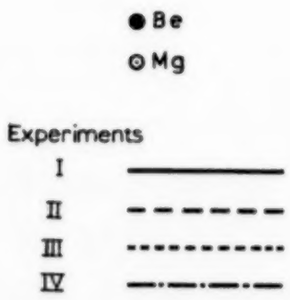


Fig. 5b



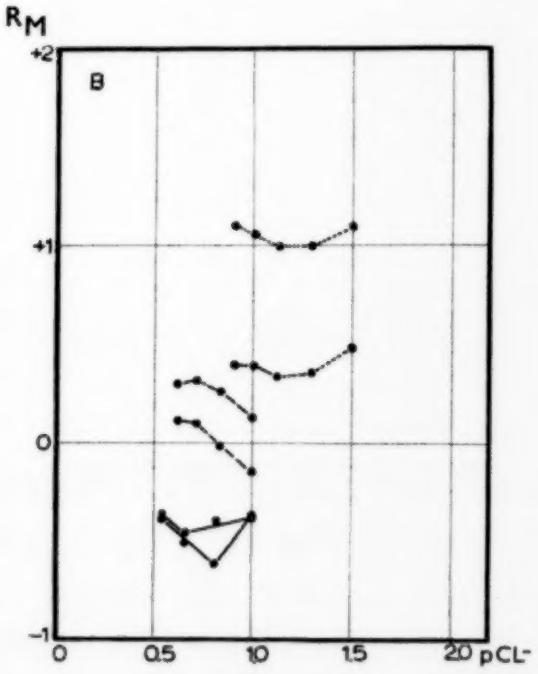


Fig. 6b

With these eluants free migration of ions is observed only if the H_2O content is higher than 10 %; optimal conditions for separation are those of Expts. A.II, A.III and B.III.

Group IIB—cadmium, zinc, mercury

The curves of these elements are all rather similar, since the behaviour of these elements resembles that of Au, from which it may be presumed that these complexes are neutral or anionic.

It may be mentioned that the frequent cross-linking of these curves indicates variations of anionic character with the composition of the eluant. However, it can be observed that with low [HCl] (less than 5 %) a sharp decrease in the R_F occurs, which must indicate a new ionic, less negative, species; this can be clearly seen in Expt. B.III.

From the shape of the curves of the R_M values, it appears that, under the conditions of Expts. A.I, a change in the ionic form occurs between 30 % and 50% H_2O. The high R_F values observed here, make it difficult to determine the variation of the R_M–pH_2O lines.

In the case of low [H_2O], the spots obtained had no tails.

There is no optimum for separation of the three elements, although the zones of 30, 40 and 50 % H_2O of Expts. A.I and the zone of 24 % H_2O of Expt. A.II must be pointed out.

Group IIIA—lanthanum, thorium

The behaviour of this group is very similar to that of Group IA. The separation of the two elements is impossible under any of the conditions used in the experiments.

Group IIIB—gallium, indium, thallium

Under the conditions of Expt. A.IV, and even more so under those of Expt. A.III, Ga seems to be anionic at low [H_2O], but becomes cationic at higher water concentrations. However, in Expts. A.I and A.II, with high [HCl], the complex is always anionic.

The fact that the Ga curves show a maximum (R_F = 1.0), could be explained by the occurrence of an intermediate zone between anionic and cationic, in which the complex is non-ionic and thus migrates with the solvent front.

Indium also exhibits curves that are quite different from those of the former elements. It must be pointed out, for instance, that when [H_2O] = 70 %, and [HCl] is lowered from 17.6 % to 11 %, the R_F of In decreases abruptly, while that of the other elements remains the same or is even increased.

The discontinuity of the curves of series A and the relative continuity of the curves of series B, proves that [H_2O] is more important than [HCl] for the R_F values.

Tl seems to form anionic or non-ionic complexes under all the experimental conditions.

Good conditions for simultaneous separation are:

(1) [H_2O] = 5.0 % and [HCl] = 0.88 %.
(2) [H_2O] = 22.0 % and [HCl] = 4.4 %.

TABLE

R_F AND R_M VALUES OF ELEMENTS NOT REPRESENTED

Elements	Eluants								
	I				II				
	1	*2*	*3*	*4*	*1*	*2*	*3*	*4*	*5*
Group IIB									
Cd	0.84	0.95	0.99	0.88	0.88	0.91	0.93	0.99	0.88
	(—0.72)	(—1.98)	(—1.99)	(0.85)	(—0.85)	(—1.96)	(—1.97)	(—1.99)	(—0.85)
Zn	0.70	0.75	0.82	0.89	0.75	0.78	0.82	0.90	0.86
	(—0.37)	(—0.47)	(—0.66)	(—0.92)	(—0.52)	(—0.55)	(—0.66)	(—0.96)	(—0.80)
Hg	0.74	0.79	0.86	0.85	0.79	0.83	0.95	0.97	0.92
	(—0.46)	(—0.57)	(—0.80)	(—0.74)	(—0.57)	(—0.70)	(—1.98)	(—1.99)	(—1.96)
Group IIIA									
La	0.09	0.26	0.54	0.79	0.04	0.08	0.17	0.43	—
	(1.00)	(0.45)	(—0.07)	(—0.57)	(1.38)	(1.06)	(0.68)	(0.12)	
Th	0.09	0.22	0.51	0.74	0.04	0.07	0.15	0.41	—
	(1.00)	(0.55)	(—0.02)	(—0.46)	(1.38)	(1.11)	(0.75)	(0.16)	
Group IIIB									
Ga	0.91	0.94	0.97	0.81	0.90	0.93	0.99	0.99	0.94
	(—0.96)	(—1.97)	(—1.99)	(—0.64)	(—0.96)	(—1.97)	(—1.99)	(—1.99)	(—1.97)
In	0.35	0.43	0.56	0.85	0.37	0.37	0.46	0.56	0.48 ↕
	(0.27)	(0.12)	(—0.10)	(—0.74)	(0.23)	(0.23)	(0.07)	(—0.10)	(0.04)
Tl	0.91	0.94	0.98	0.88	0.91	0.96	1.00	1.00	0.96
	(—0.96)	(—1.97)	(—1.99)	(—0.85)	(—0.96)	(—1.98)	(—∞)	(—∞)	(—1.98)
Group IVB									
Ge	—	—	—	—	0.95	0.97	0.90	0.90	—
					(—1.98)	(—1.99)	(—0.96)	(—0.96)	
Sn	0.78	0.82	0.89	0.84	0.81	0.83	0.90	0.97	—
	(—0.55)	(—0.66)	(—0.92)	(—0.72)	(—0.64)	(—0.70)	(—0.96)	(—1.99)	
Pb	0.30	0.41	0.61	0.81	0.16 ↕	0.21 ↕	0.34	0.51	—
	(0.37)	(0.16)	(—0.19)	(—0.64)	(0.72)	(0.56)	(0.29)	(—0.02)	
Group V A									
V	0.26	0.39	0.60	0.80	0.19	0.27	0.37	0.53	—
	(0.45)	(0.20)	(—0.17)	(0.60)	(0.62)	(0.43)	(0.23)	(—0.05)	
Group V B									
Sb	—	—	—	—	0.75	0.83	0.95	0.97	0.89
					(—0.47)	(—0.70)	(—1.98)	(—1.99)	(—0.92)
Bi	0.47	0.57	0.70	0.95	0.48	0.56	0.68	0.77	0.84
	(0.05)	(—0.12)	(—0.37)	(—1.98)	(0.04)	(—0.10)	(—0.33)	(—0.52)	(—0.72)
Group VI A									
Cr	0.18	0.24	0.46	0.85	0.06	0.13	0.22	0.41	0.85
	(0.65)	(0.50)	(0.07)	(—0.74)	(1.18)	(0.82)	(0.55)	(0.16)	(—0.74)
Group VI B									
Se	0.93	0.93	0.88 ↕	0.84	0.87	0.84	0.71	0.77 ↑	0.89
	(—1.97)	(—1.97)	(—0.85)	(—0.72)	(—0.82)	(—0.72)	(—0.39)	(—0.52)	(—0.92)
Te	0.81	0.86	0.88	0.80	0.85	0.86	0.90	0.93	0.85
	(—0.64)	(—0.80)	(—0.85)	(—0.60)	(—0.74)	(—0.80)	(—0.96)	(—1.97)	(—0.74)
Group VII A									
Mn	0.26	0.37	0.60	0.90	0.13	0.18	0.32	0.52	—
	(0.45)	(0.23)	(—0.17)	(—0.96)	(0.82)	(0.65)	(0.33)	(—0.03)	
Re	0.73	0.79	0.85	0.88	0.81	0.82	0.91	0.99	—
	(—0.43)	(—0.57)	(—0.74)	(—0.85)	(—0.64)	(—0.66)	(—0.96)	(—1.99)	
Group VIII									
Fe	0.92	0.95	0.97	0.82	0.93	0.94	0.96	0.96	0.87
	(—1.96)	(—1.98)	(—1.99)	(—0.66)	(—1.97)	(—1.97)	(—1.98)	(—1.98)	(—0.82)
	—	—	—	—	—	—	—	—	—
Pt	0.63	0.67	0.72	0.81	0.69	0.74	0.81	0.90	0.68
	(—0.23)	(—0.31)	(—0.41)	(—0.64)	(—0.35)	(—0.46)	(—0.64)	(—0.96)	(—0.33)
	0.47	0.52	0.62	0.81	0.52	0.58	0.67	0.76	0.68
	(0.05)	(—0.03)	(—0.21)	(—0.64)	(—0.03)	(—0.13)	(—0.31)	(—0.49)	(—0.33)

II

GRAPHICALLY (SERIES A) (R_M values are given in parentheses)

Eluants									
III					*IV*				
1	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	*5*
0.92	0.93	0.93	0.92	0.87	—	0.55↓	0.57↓	—	0.56↓
(—1.96)	(—1.97)	(—1.97)	(—1.96)	(—0.82)		(—0.07)	(—0.12)		(—0.10)
0.86	0.87	0.91	0.94	0.91	0.70↓	0.67↓	0.62↓	0.59↓	0.56↓
(—0.80)	(—0.82)	(—0.96)	(—1.97)	(—0.96)	(—0.37)	(—0.31)	(—0.21)	(—0.15)	(—0.10)
0.87	0.93	0.97	1.00	0.99	0.60↓	—	0.60↓	0.57↓	0.63
(—0.82)	(—1.97)	(—1.99)	(—∞)	(—1.99)	(—0.17)		(—0.17)	(—0.12)	(—0.23)
0	0	0	0.02	0.08	0	0	0	0	0
(+∞)	(+∞)	(+∞)	(1.69)	(1.06)	(+∞)	(+∞)	(+∞)	(+∞)	(+∞)
0	0	0	0.02	0.06	0	0	0	0	0
(+∞)	(+∞)	(+∞)	(1.69)	(1.18)	(+∞)	(+∞)	(+∞)	(+∞)	(+∞)
0.96	1.00	0.99	0.87	0.40↓	0.82↓	0.77↓	0.78↓	0.73	0.53
(—1.98)	(—∞)	(—1.99)	(—0.82)	(0.18)	(—0.66)	(—0.52)	(—0.55)	(—0.43)	(—0.05)
0.46	0.43	0.36	0.36	0.39	0.67↓	—	—	0.60	0.22
(0.07)	(0.12)	(0.25)	(0.25)	(0.20)	(—0.31)			(—0.17)	(0.55)
0.98	0.99	0.99	0.99	0.99	0.91↓	0.92↓	0.91↓	0.93↓	0.84
(—1.99)	(—1.99)	(—1.99)	(—1.99)	(—1.99)	(—0.96)	(—1.96)	(—0.96)	(—1.97)	(—0.72)
0.74↕	0.65	0.58	0.54	0.48	—	—	—	—	0.23
(—0.46)	(—0.27)	(—0.13)	(—0.07)	(0.04)					(0.53)
0.94	0.97	0.98	0.98	0.95	—	—	—	—	—
(—1.97)	(—1.99)	(—1.99)	(—1.99)	(—1.98)					
0.13↓	0.17↓	0.20↓	0.25↓	0.31↓	0	0	0	0	0
(0.82)	(0.68)	(0.60)	(0.48)	(0.35)	(+∞)	(+∞)	(+∞)	(+∞)	(+∞)
—	0.08	0.13	0.19	0.23	—	—	—	—	—
	(1.06)	(0.82)	(0.62)	(0.53)					
0.90↓	0.90↓	0.93	0.94	0.94	0	0	0	0	0
(—0.96)	(—0.96)	(—1.97)	(—1.97)	(—1.97)	(+∞)	(+∞)	(+∞)	(+∞)	(+∞)
—	0.71↓	0.71	0.69	0.75	0	0	0	0	0
	(—0.39)	(—0.39)	(—0.35)	(—0.47)	(+∞)	(+∞)	(+∞)	(+∞)	(+∞)
—	0.03	0.05	0.08	—	0↑	0↑	0↑	0↑	0↑
	(1.51)	(1.28)	(1.06)		(+∞)	(+∞)	(+∞)	(+∞)	(+∞)
0.62↕	0.68↕	0.69	0.75	0.76	0.73↑	0.70↑	0.68↑	0.55↕	0.54
(—0.21)	(—0.33)	(—0.35)	(—0.47)	(—0.49)	(—0.43)	(—0.37)	(—0.33)	(—0.09)	(—0.07)
0.86↓	0.88↓	0.83	0.81	0.60	0.52↑	0.49↑	0.49↑	0.48↑	0.13↕
(—0.80)	(—0.85)	(—0.70)	(—0.64)	(—0.17)	(—0.03)	(0.02)	(0.02)	(0.04)	(0.82)
0.06	0.05	0.06	0.11	0.17	—	—	—	—	—
(1.18)	(1.28)	(1.18)	(0.90)	(0.68)					
0.88	0.90	0.95	0.99	1.00	0.68↓	0.67↓	0.66↓	—	0.73↓
(—0.85)	(—0.96)	(—1.98)	(—1.99)	(—∞)	(—0.33)	(—0.31)	(—0.28)		(—0.43)
0.95	0.97	0.85	0.67	0.22	0.68↓	0.63↓	—	—	0.26↓
(—1.98)	(—1.99)	(—0.74)	(—0.31)	(0.55)	(—0.33)	(—0.23)			(0.45)
—	—	0.05	0.08	0.15	—	—	—	—	—
		(1.28)	(1.06)	(0.75)					
0.91	0.89	0.86	0.90	0.91	—	—	—	0.58↓	0.61↓
(—0.96)	(—0.92)	(—0.80)	(—0.96)	(—0.96)				(—0.13)	(—0.19)
0.53	0.60	0.66	0.74	0.78	—	—	—	—	—
(—0.05)	(—0.17)	(—0.28)	(—0.46)	(—0.55)					

R_F AND R_M VALUES OF ELEMENTS NOT REPRESENTED

Elements	*Eluants*									
	I					*II*				
	1	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	5
Group II B										
Cd	—	0.81	0.87	0.94	0.96	0.61	0.51	0.86	0.96	—
		(—0.64)	(—0.82)	(—1.97)	(—1.98)	(—0.19)	(—0.02)	(—0.80)	(—1.98)	
Zn	—	0.71	0.83	0.94	0.96	0.67	0.74	0.81	0.91	—
		(—0.39)	(—0.70)	(—1.97)	(—1.98)	(—0.31)	(—0.46)	(—0.64)	(—0.96)	
Hg	—	0.70	0.86	0.99	0.98	0.66	0.74	0.88	0.97	—
		(—0.37)	(—0.80)	(—1.99)	(—1.99)	(—0.28)	(—0.46)	(—0.85)	(—1.99)	
Group III A										
La	—	0.57	0.63	0.58	0.60	0.17	0.20	0.21	0.30	—
		(—0.12)	(—0.23)	(—0.13)	(—0.17)	(0.68)	(0.60)	(0.58)	(0.37)	
Th	—	0.53	0.58	0.58	0.69	0.15	0.15	0.19	0.28	—
		(—0.05)	(—0.13)	(—0.13)	(—0.35)	(0.75)	(0.75)	(0.62)	(0.41)	
Group III B										
Ga	—	0.99	1.00	1.00	0.71 ↓	0.97	0.97	0.98	1.00	—
		(—1.99)	(—∞)	(—∞)	(—0.39)	(—1.99)	(—1.99)	(—1.99)	(—∞)	
In	—	0.68	0.72	0.75	0.58	0.42	0.44	0.50	0.54	—
		(—0.33)	(—0.41)	(—0.47)	(—0.13)	(0.14)	(0.10)	(0)	(—0.07)	
Tl	—	0.82 ↑	1.00	1.00	1.00	0.88	0.97	0.98	1.00	—
		(—0.66)	(—∞)	(—∞)	(—∞)	(—0.85)	(—1.99)	(—1.99)	(—∞)	
Group IV B										
Ge	—	—	—	—	—	0.98	0.98	0.98	0.95	—
						(—1.99)	(—1.99)	(—1.99)	(—1.98)	
Sn	—	0.74	0.86	1.00	1.00	0.66	0.74	0.85	0.94	—
		(—0.46)	(—0.80)	(—∞)	(—∞)	(—0.28)	(—0.46)	(—0.74)	(—1.97)	
Pb	—	0.73	0.73	0.70	0.33	0.39	0.38	0.39	0.39	—
		(—0.43)	(—0.43)	(—0.37)	(—0.43)	(0.20)	(0.21)	(0.20)	(0.20)	
Group V A										
V	—	0.63	0.73	0.70	0.52	0.36	0.38	0.43	0.48	—
		(—0.23)	(—0.43)	(—0.37)	(—0.03)	(0.25)	(0.21)	(0.12)	(0.04)	
Group V B										
Sb	—	0.77	0.79	0.91	0.98	0.63	0.68	0.83	0.94	—
		(—0.52)	(—0.57)	(—0.96)	(—1.99)	(—0.23)	(—0.33)	(—0.70)	(—1.97)	
Bi	—	0.73	0.81	0.88	0.79	0.47	0.55	0.64	0.75	—
		(—0.43)	(—0.64)	(—0.85)	(—0.57)	(0.05)	(—0.09)	(—0.25)	(—0.47)	
Group VI A										
Cr	—	0.59	0.65	0.72	0.57	0.26	0.28	0.33	0.37	—
		(—0.15)	(—0.27)	(—0.41)	(—0.12)	(0.45)	(0.41)	(0.31)	(0.23)	
Group VI B										
Se	—	0.81	0.79	0.81	0.66 ↕	0.89	0.88	0.69	0.77	—
		(—0.64)	(—0.57)	(—0.64)	(—0.28)	(—0.92)	(—0.85)	(—0.35)	(—0.52)	
Te	—	0.67	0.79	0.81	0.67	0.73	0.83	0.87	0.95	—
		(—0.31)	(—0.57)	(—0.64)	(—0.31)	(—0.43)	(—0.70)	(—0.82)	(—1.98)	
Group VII A										
Mn	—	0.67	0.71	0.75	0.60	0.33	0.31	0.38	0.42	—
		(—0.31)	(—0.39)	(—0.47)	(—0.17)	(0.31)	(0.35)	(0.21)	(0.14)	
Re	—	0.74	0.82	0.88	1.00	0.67	0.74	0.88	0.98	—
		(—0.46)	(—0.66)	(—0.85)	(—∞)	(—0.31)	(—0.46)	(—0.85)	(—1.99)	
Group VIII										
Fe	—	0.80	0.87	0.78	0.61	0.93	0.94	0.95	0.97	—
		(—0.60)	(—0.82)	(—0.55)	(—0.19)	(—1.97)	(—1.97)	(—1.98)	(—1.99)	
	—	—	—	—	—	—	—	—	—	—
Pt	—	0.61	0.74	0.86	0.98	0.55	0.68	0.78	0.88	—
		(—0.19)	(—0.46)	(—0.80)	(—1.99)	(—0.09)	(—0.33)	(—0.55)	(—0.85)	
	—	—	—	—	—	0.42	0.50	0.68	0.79	—
						(0.14)	(0)	(—0.33)	(—0.57)	

III

GRAPHICALLY (SERIES B) (R_M values are given in parentheses)

Eluants									
III					*IV*				
1	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	*5*
0.83 (—0.70)	0.87 (—0.82)	0.95 (—1.98)	0.97 (—1.99)	0.79 (—0.57)	0.76↓ (—0.49)	0.66↓ (—0.28)	0.62↓ (—0.21)	0.50↓ (0)	0.45↓ (0.09)
0.73 (—0.43)	0.80 (—0.60)	0.90 (—0.96)	0.90 (—0.96)	0.72 (—0.41)	—	—	—	—	—
0.76 (—0.49)	0.88 (—0.85)	0.97 (—1.99)	0.98 (—1.99)	0.82 (—0.66)	0.82↓ (—0.66)	0.72↓ (—0.41)	0.67↓ (—0.31)	0.60↓ (—0.17)	0.59↓ (—0.15)
0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0 (+∞)	0 (+∞)	0 (+∞)	0 (+∞)	0 (+∞)
0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0 (+∞)	0 (+∞)	0 (+∞)	0 (+∞)	0 (+∞)
0.93 (—1.97)	0.98 (—1.99)	1.00 (—∞)	0.79 (—0.57)	0.26↓ (0.45)	0.95↓ (—1.98)	0.94↓ (—1.97)	0.90↓ (—0.96)	0.80↓ (—0.60)	0.75↓ (—0.47)
0.34 (0.29)	0.43 (0.12)	0.42 (0.14)	0.36 (0.25)	0.34 (0.29)	—	—	0.36↕ (0.25)	0.29↕ (0.39)	0.19↕ (0.62)
0.90 (—0.96)	0.96 (—1.98)	0.98 (—1.99)	0.99 (—1.99)	0.99 (—1.99)	1.00↓ (—∞)	1.00↓ (—∞)	1.00↓ (—∞)	0.93↓ (—1.97)	0.85↓ (—0.74)
0.71 (—0.39)	0.90 (—0.96)	0.69 (—0.35)	0.56 (—0.10)	0.38 (0.21)	0.62 (—0.21)	0.47 (0.05)	0.36 (0.25)	0.31 (0.35)	0.26 (0.45)
0.68 (—0.33)	0.84 (—0.72)	0.91 (—0.96)	0.95 (—1.98)	0.83 (—0.70)	1.00↓ (—∞)	0.99↓ (—1.99)	0.96↓ (—1.98)	0.75↓ (—0.47)	0.54↓ (—0.07)
0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0 (+∞)	0 (+∞)	0 (+∞)	0 (+∞)	0 (+∞)
0.20 (0.60)	0.26 (0.45)	0.29 (0.40)	0.24 (0.50)	0.15 (0.75)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)
0.74 (—0.46)	0.84 (—0.72)	0.90 (—0.96)	0.96 (—1.98)	0.82 (—0.66)	0.85↓ (—0.74)	0.73↓ (—0.43)	0.72↓ (—0.41)	0.64↓ (—0.25)	0.63↓ (—0.23)
0.45 (0.09)	0.58 (—0.13)	0.65 (—0.27)	0.72 (—0.40)	0.65 (—0.27)	0.80↓ (—0.60)	0.78↓ (—0.55)	0.74↓ (—0.46)	0.56↓ (—0.10)	0.50↓ (0)
0.04 (1.38)	0.07 (1.11)	0.07 (1.11)	0.09 (1.00)	0.07 (1.11)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)	0↑ (+∞)
0.60 (—0.17)	0.64 (—0.25)	0.73 (—0.43)	0.74 (—0.46)	0.64 (—0.25)	0.83 (—0.70)	0.74 (—0.46)	0.56 (—0.10)	0.50 (0)	0.46 (0.07)
0.82 (—0.66)	0.85 (—0.74)	0.91 (—0.96)	0.79 (—0.57)	0.39 (0.20)	0.80 (—0.60)	0.67↓ (—0.31)	0.48↓ (0.04)	0.29↓ (0.39)	0.11↓ (0.90)
0.11 (0.90)	0.11 (0.90)	0.12 (0.86)	0.12 (0.86)	0.09 (1.00)	—	—	—	—	—
0.80 (—0.60)	0.84 (—0.72)	0.98 (—1.99)	1.00 (—∞)	0.95 (—1.98)	0.86↓ (—0.80)	0.79↓ (—0.57)	0.74↓ (—0.46)	0.59 (—0.15)	0.54 (—0.07)
0.91 (—0.96)	0.90 (—0.96)	0.85 (—0.74)	0.56 (—0.10)	0.24 (0.50)	0.98 (—1.99)	0.83 (—0.70)	0.70↓ (—0.37)	0.57↓ (—0.12)	0.49↓ (0.02)
—	—	—	—	—	—	—	—	—	—
0.69 (—0.35)	0.81 (—0.64)	0.93 (—1.97)	0.92 (—1.96)	0.77 (—0.52)	0.91 (—0.96)	0.76 (—0.49)	0.70↓ (—0.37)	0.63↓ (—0.23)	0.51↓ (—0.02)
0.53 (—0.05)	0.65 (—0.27)	0.75 (—0.47)	0.73 (—0.43)	0.63 (—0.23)	—	—	—	—	—

Group IVA—titanium, zirconium

The migration of these two elements, like that of the A groups described above, is chiefly determined by the $[H_2O]$ of the eluant.

However, the behaviour of Ti observed in Fig. 7a and 7b, shows that the ionic form existing at low $[H_2O]$ ($< 25\%$) is different from that existing at higher concentrations.

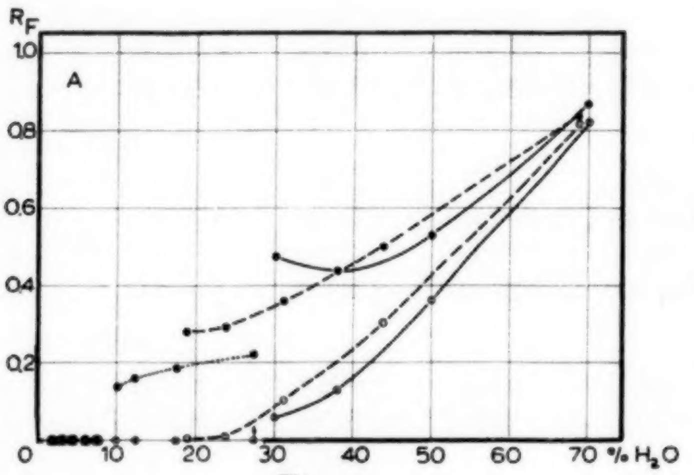


Fig. 7a

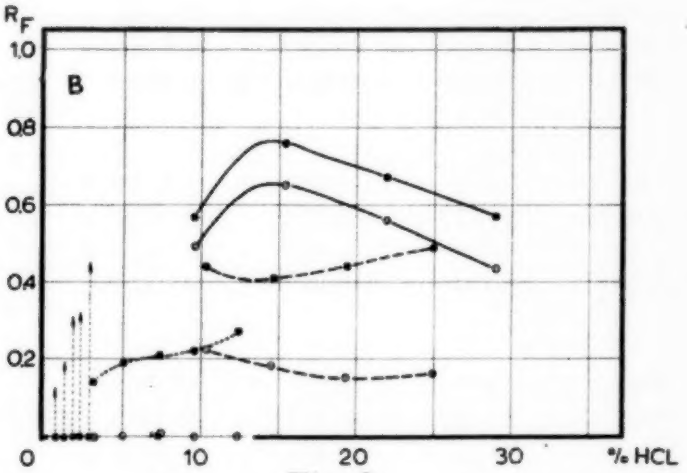


Fig. 8a

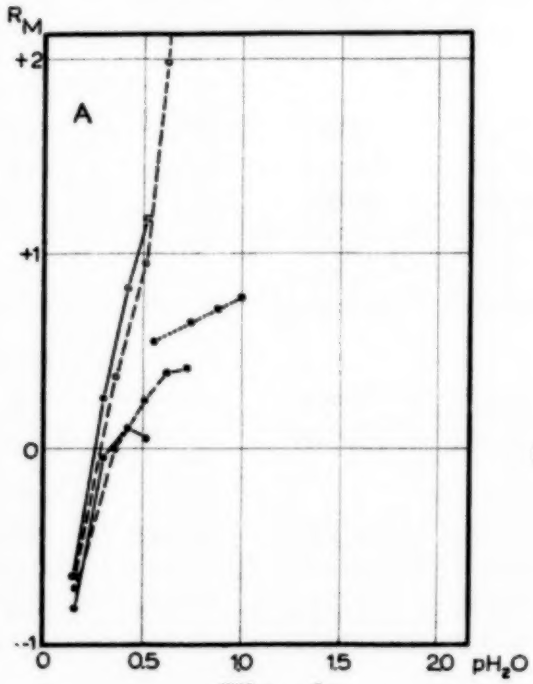
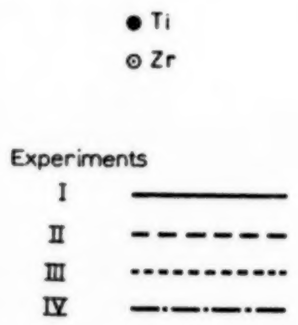


Fig. 7b



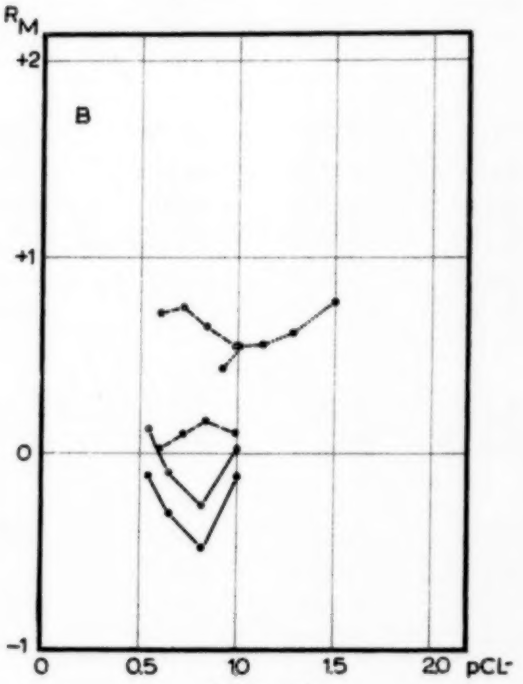


Fig. 8b

It can be observed in Fig. 8b that the ionic form varies in Expts. B.I and B.II.

There is great similarity between the curves of these elements (chiefly Ti) and those of Group IIA elements (chiefly Be).

Good conditions for the separation of the two elements are those of:

(1) Expts. A.I and A.II (up to $[H_2O] = 50\%$).

(2) Expts. A.III, B.II and B.III.

The best one ($\Delta R_F = 0.40$) is: $[HCl] = 17.6\%$ and $[H_2O] = 30\%$.

Group IVB—germanium, tin, lead

Ge exhibits curves that differ from those of the former B sub-groups. It seems cationic at low $[H_2O]$ and $[HCl]$, and anionic or non-ionic at high $[H_2O]$ and $[HCl]$. In Expts. B.III and B.IV, the presence of two ionic species is clearly observed.

The curves for Sn are very similar to those of elements of Group IIB (Cd,Zn,Hg). The only important difference is evident in Expt. A.I.

The curves for Pb are similar to those of Mg, Be, etc, except in the case of eluants with low H_2O content, where low solubility alters the R_F values.

It is curious to note that under the conditions of Expt. B.I (60% H_2O), there is a distinct change of ionic form on passing from 10 to 20% HCl. Further increases of [HCl] have no affect on the R_F value.

Optimal conditions of separation for these elements are:

(1) $[H_2O] = 15$ to 20% and [HCl] = 4 to 5%.
(2) $[H_2O] = 40\%$ and [HCl] = 25%.

Group VA—vanadium

The behaviour of V is very similar to that of Ti and Be.

The variation of ionic species that Be and Ti exhibit in Expt. A.III, is not so distinct in the case of V. On the other hand, under the conditions of Expt. B.III a distinct variation of ionic form occurs, which is not very marked with Ti and Be.

Group VB—antimony, bismuth

The curves of both these elements are very similar to those of Sn, except that the curves of Bi are at a "lower" level of R_F.

Interpretation of the R_M curve is difficult in the case of Sb, since the R_F values are very high.

In Expt. A.III the ionic form of Bi is quite different from those in Expts. A.I and A.II. In Expts. B.I and B.III also, a change is observed in the ionic species.

Good conditions for separation are:

$[H_2O] = 10$ to 30% and [HCl] = 4 to 11%.

Group VIA—chromium, molybdenum, uranium

Cr was not included in Figs. 9a and 10a in order to avoid overcrowding the graphs; U was chromatographed in valencies (IV) and (VI).

The behaviour of Cr is very similar to that of V.

In Fig. 9a Mo shows a behaviour that is similar to that of Ge, and in Fig. 10a, to that of Sn. In Figs. 9b and 10b two different forms can be clearly seen.

The behaviour of U (IV) is very similar to that of La, Th, etc., only one form appearing in Fig. 9b and two forms in Fig. 10b.

U (VI) behaves quite differently, being very similar to Cu. As can be observed in Figs. 9b and 10b, there must be a succession of ionic forms, since the changes of direction of the curves are not abrupt but gradual.

The best conditions for separation of the three species are:

$[H_2O] = 7$ to 40% and [HCl] = 5 to 17%.

Group VIB—selenium, tellurium

The curves of Se and Te have curious shapes with frequent crossings and shifts. The R_F values are always high for Se, but for Te they are low at low water and acid

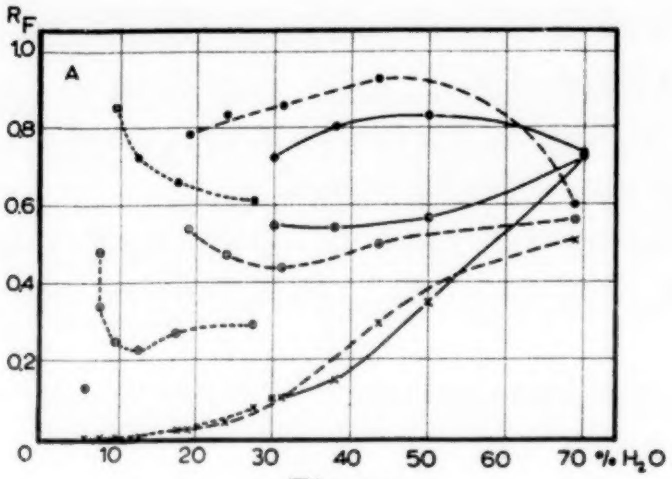


Fig. 9a

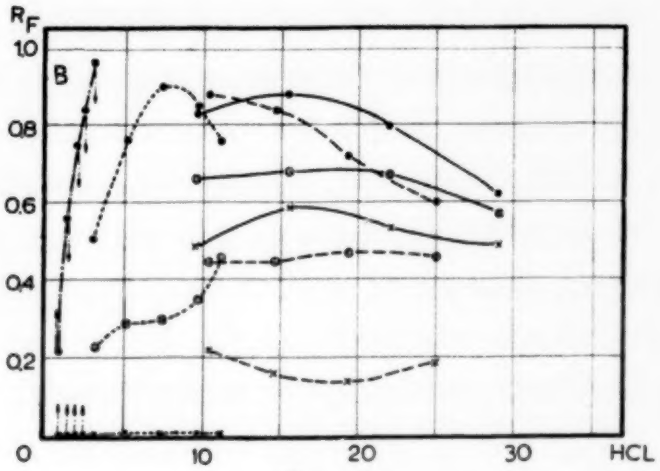


Fig. 10a

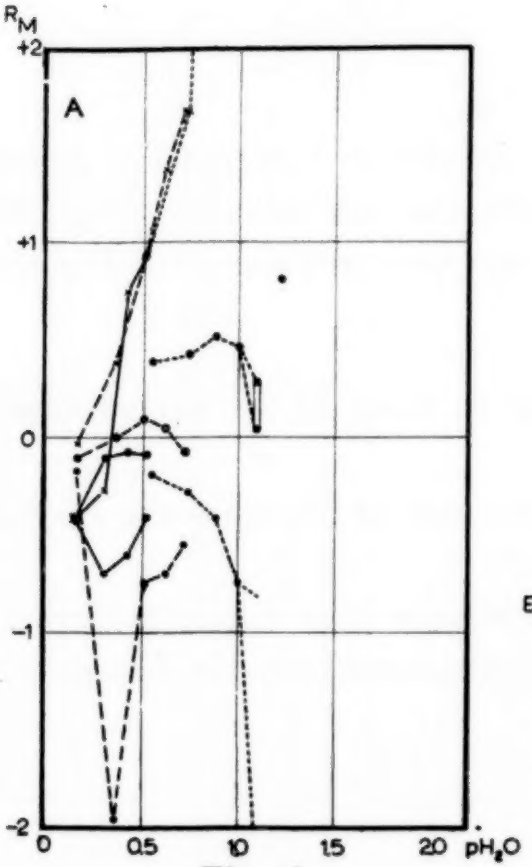
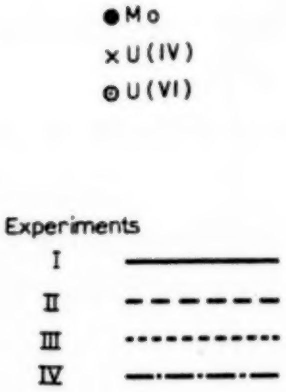


Fig. 9b



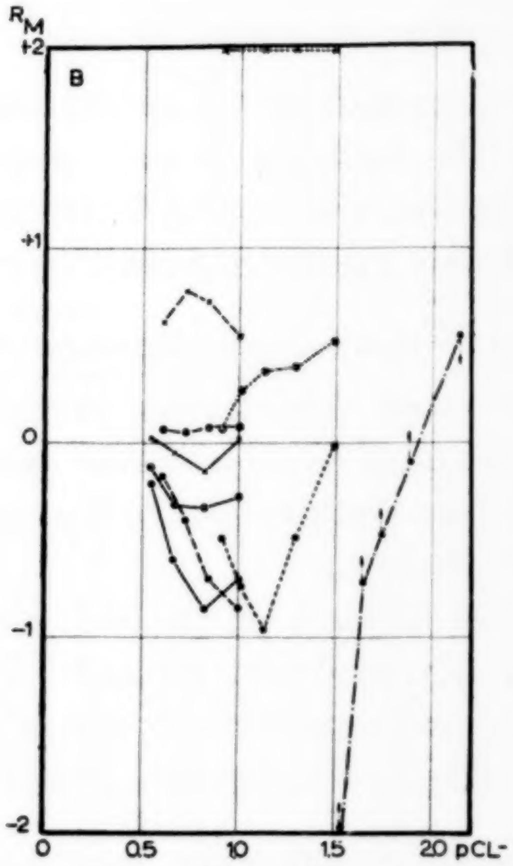


Fig. 10b

concentration, which perhaps indicates a cationic form. As is clearly shown by the graphs, Te exhibits two ionic forms and Se possibly three.

The best conditions for separation are:

$[H_2O] = 5\%$ and $[HCl] = 1\%$

$[H_2O] = 20\%$ and $[HCl] = 2\%$

$[H_2O] = 20\%$ and $[HCl] = 7$ to 12%.

Group VIIA—rhenium, manganese

Mn presents curves that are characteristic for cationic species, being similar to those of La, Mg, etc.

Re, on the other hand, exhibits curves that are characteristic for anionic forms, being similar to those of Sn and Mo.

In graphs it can be seen that both Mn and Re present two ionic forms.

A broad zone of separation is observed at:

$[H_2O] = 4$ to 40% and $[HCl] = 5$ to 25%.

Group VIII—cobalt, nickel, iron, platinum

In order to avoid overcrowding the graphs, only Co and Ni are represented in Figs. 11a and 12a.

Ni presents curves that are similar to those of other elements known to be cationic. According to Fig. 11b it has only one ionic form, and according to Fig. 12b, two forms.

The behaviour of Co is very similar to that of Ni in certain zones, but quite different in other zones. The R_F values of Co are always greater than those of Ni, which probably signifies more anionic complexes. It can be noted in all the curves presented that the acid concentration has a greater influence than the water concentration.

The element that is most similar to Co in chromatographic behaviour, is Cu.

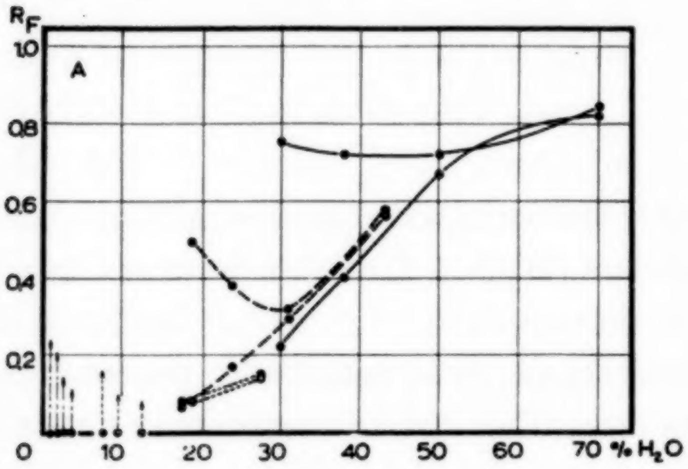


Fig. 11a

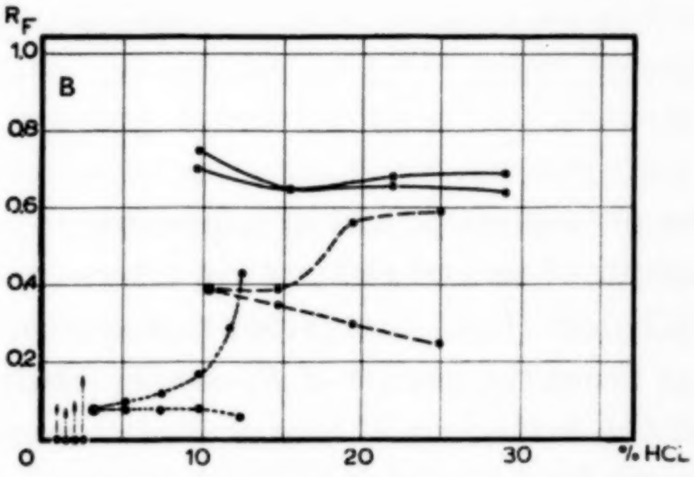


Fig. 12a

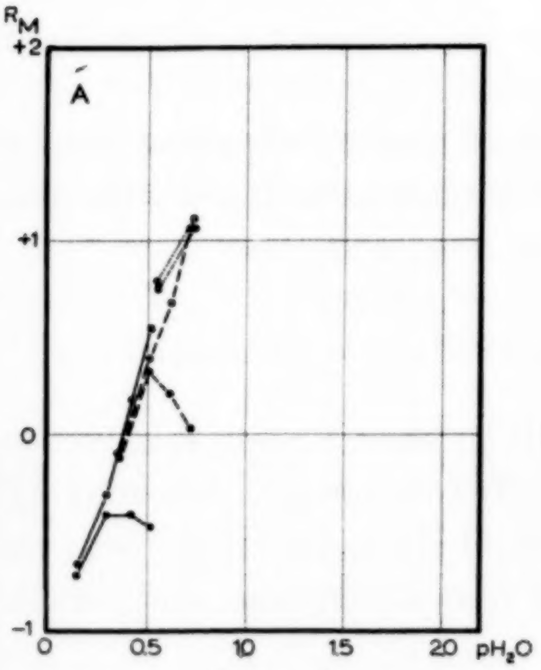
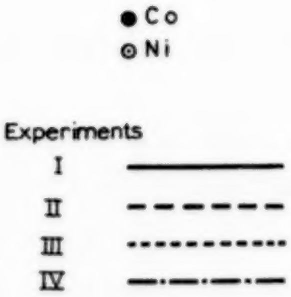


Fig. 11b



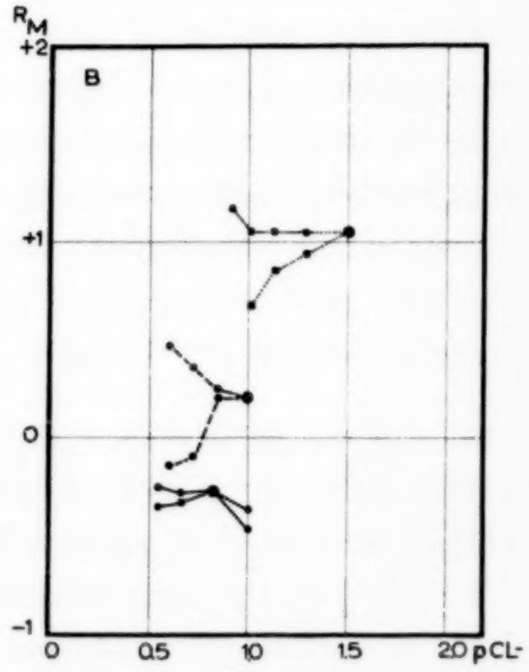


Fig. 12b

The presence of two ionic species is clearly observed in Fig. 11b and perhaps three in Fig. 12b.

The best conditions for separation are:

$[H_2O] = 40\%$ and $[HCl] = 25\%$
$[H_2O] = 30\%$ and $[HCl] = 17\%$
$[H_2O] = 17$ to 20% and $[HCl] = 11$ to 12%.

Both Fe and Pt exhibit two spots in several zones. These spots are very far from one another with eluants of low [HCl] and [H_2O]. In the zone with 10% water and 4% hydrochloric acid, it seems clear that there are two complexes, probably one cationic and the other anionic. It is curious to observe that in the case of Pt the spots of lower R_F seem to be of the same ionic species, as can be inferred from the parallelism of the lines of R_M *vs.* pH_2O referring to these complexes. However, with the complexes of higher R_F, two or three ionic forms seem to be present.

The similarity in behaviour of Pt and Sn must be pointed out.

In the case of Fe, there appear to be three ionic forms.

Good separation of these two metals can be obtained under the following conditions:

[H_2O] = 20 to 30% and [HCl] = 4.4%
[H_2O] = 40% and [HCl] = 20 to 25%.

CONCLUSIONS

The present work makes it possible to check the similarity in chromatographic behaviour of several elements in a ternary solvent (H_2O + HCl + *n*-butanol) in which [HCl] and [H_2O] are varied in a systematic manner.

From the curves of R_F *versus* [HCl] and [H_2O], it is possible to see whether or not the behaviour of two or more elements is similar and to establish the best conditions for separation of these elements.

By plotting curves of R_M *versus* pH_2O and pCl^- an attempt can be made to determine the number of ionic species exhibited by each element and the similarity to ionic species of other elements.

An interesting application of the results obtained is the indications that these curves give when separations by ion exchange are desired. In this case a zone must be chosen in which one element has a high R_F and the other a low one.

SUMMARY

Several curves of R_F *vs.* [H_2O] and R_F *vs.* [HCl] were traced relating to 33 elements, using mixtures of *n*-butanol + H_2O + HCl as eluants, whereby [H_2O] (or [HCl]) was fixed at various levels and at each level [HCl] (or [H_2O]) was varied.

In this way the best conditions for separation were determined and similarities among the various elements were established.

Several curves of R_M *vs.* pH_2O and R_M *vs.* pCl^- were traced and from these some conclusions as to the ionic species present were drawn.

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CHROMATOGRAPHY OF PHENOLIC COMPOUNDS ON BORATE IMPREGNATED PAPER

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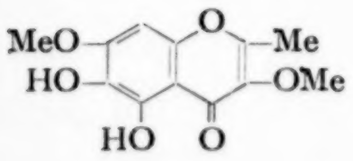
Phenols which contain *ortho*-dihydroxy or *ortho*-hydroxy-carboxy groupings readily form borate complexes. In organic solvents, therefore, these phenols have lower R_F values on paper impregnated with sodium borate than on plain paper or paper impregnated with sodium phosphate. This property forms the basis of a chromatographic test[1,2] which is being increasingly employed as a means for the detection of such groupings in both natural and synthetic polyphenols. In some instances, conclusive confirmatory evidence for the presence of a catechol nucleus has been obtained from chemical data, *e.g.* in pristimerol[3]. In other recent cases, however, a decision in favor of one of two or more alternate structures has been made primarily on the basis of the retarded migration of the compound on borate paper. Thus, of two possible structures (I) and (II) for the product of a Kostanecki acylation of 3,6-dihydroxy-ω,2,4-trimethoxy-acetophenone, (I) was adopted as a result of its slow movement on borate paper[4].

For similar reasons, the product of the metabolism of hydroxyhydroquinone by the broad bean was thought to be probably 3,4-dihydroxyphenyl-β-glucoside[5] while the lichen constituent, porphyrilic acid, was considered to be either (III) or (IV), an alternate structure (V) being excluded at first from its low R_F value on borate paper[6]. Subsequently, it was shown that the correct constitution of porphyrilic acid was (V).

The examples quoted above indicate the extent to which the borate paper test is



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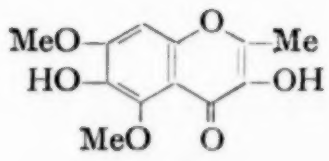

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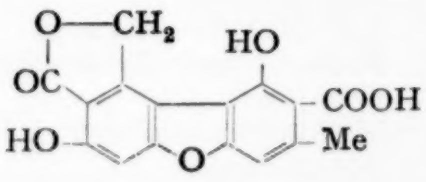

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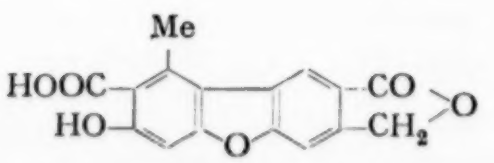

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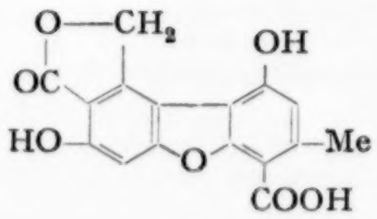

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being applied in structural analysis. For this reason it now seems to be of some importance to present additional chromatographic and spectrophotometric evidence, obtained some years ago, which shows that retardation on sodium borate paper is a property shared by the majority of phenolic compounds and is not confined to those which contain particular chelatogenic groupings.

In the procedure described, the R_F values of the phenol are determined on papers impregnated with 0.1 M $NaBO_3$ and with 0.1 M Na_2HPO_4. Lower R_F values on the borate paper were reported to indicate the presence of chelatogenic groupings such as *ortho*-dihydroxyl. It was tacitly assumed that phenols lacking such chelatogenic groupings have approximately identical R_F values on both papers. The data presented[2], however, do not support this conclusion. Thus, for example, 7-hydroxy-, 6-methoxy-7-hydroxy- and 6,7-dihydroxy-coumarin are all retarded on borate paper, the differences in the R_F values on the two papers being 0.18, 0.31 and 0.18 respectively in *n*-butanol and 0.16, 0.43 and 0.14 respectively in ethyl acetate. It is apparent that the monohydroxy-coumarins are retarded to an even greater extent than the *ortho*-dihydroxy compound. Many other phenols, which from spectrophotometric measurements are well known not to form complexes with borate[7,8], also have very low

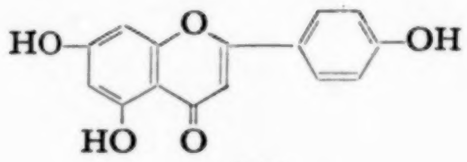

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TABLE I

INFLUENCE OF SODIUM PHOSPHATE (Na_2HPO_4) AND SODIUM BORATE ($NaBO_3$) ON SPECTRA OF PHENOLS IN *n*-BUTANOL SATURATED WITH WATER

Phenol	λ_{max} (mμ)		
	n-BuOH/H₂O	*n-BuOH/H₂O + Na₂HPO₄*	*n-BuOH/H₂O + NaBO₃*
Phloroglucinol	266	270	346, 275, 253
Salicylic acid	303	296	296, 265
p-Hydroxybenzoic acid	256	246	247
Methyl *p*-hydroxybenzoate	258	258	300, 270
p-Coumaric acid	311	285	265*
Methyl *p*-coumarate	315	315	366, 311, 265
Ferulic acid	323	310, 289	265*
Caffeic acid	325, 303	314, 286	320, 265*
Ethyl caffeate	330, 245	330	353, 264
o-Hydroxyacetophenone	326, 253	327, 254	327, 254
p-Hydroxyacetophenone	281	280	330
p-Hydroxybenzal-acetone	328	329	395
Apigenin	342, 270	360, 280	390, 275
Apigenin 7-rhamnoglucoside	341, 287	346, 288	399, 270
4′-O-Methylapigenin	333, 282	365, 281	375, 278
Tricin	350	369, 280	404, 273
Luteolin	352, 290	375, 280	386, 257
2′,4-Dihydroxy-3-methoxychalcone	388, 280	389	475, 280

* Inflection.

R_F values on borate paper, *e.g.* apigenin (VI), R_F 0.46, 0.84, 0.88 on borate, phosphate and plain paper respectively[9].

The chromatographic data suggest that in organic solvents sodium borate is a much stronger base than disodium hydrogen phosphate and consequently ionizes many phenols more extensively. Comparison of R_F values on paper impregnated with these two salts, therefore, cannot give useful structural information with more complex phenols since retardation on the borate paper may arise from *either* chelate formation *or* ionization of the phenol. Support for this conclusion has been obtained by determining the influence of Na_2HPO_4 and $NaBO_3$ on the spectra of a variety of phenols in *n*-butanol saturated with water (the solvent used in the chromatographic test). These spectral results are collected in Table I and illustrated with *p*-hydroxyacetophenone in Fig. 1. It is well established[10] that ionization of a phenolic hydroxyl produces a bathochromic shift of the λ_{max}, while ionization of a carboxyl group causes a hypsochromic shift (10–20 mμ) of the λ_{max}[11]. From Table I it is apparent that Na_2HPO_4 does not essentially affect the spectra of simple phenols, phenolic esters and phenolic ketones. The hydroxyl groups in these compounds are, therefore, not ionized by this salt. As would be anticipated, the phenolic acids showed hypsochromic shifts in the presence of Na_2HPO_4, indicating ionization of the carboxyl groups of these acids. With more highly conjugated compounds containing one or more fairly acidic hydroxyl groups, *e.g.*, the 7- and 4′-hydroxyls of apigenin and tricin, Na_2HPO_4 gave bathochromic shifts of 20–30 mμ indicating selective ionization of these hydroxyl groups. Unlike Na_2HPO_4, sodium borate produces a large bathochromic shift in the spectra of *all* of the phenols examined with the exception of *o*- and *p*-hydroxybenzoic acid

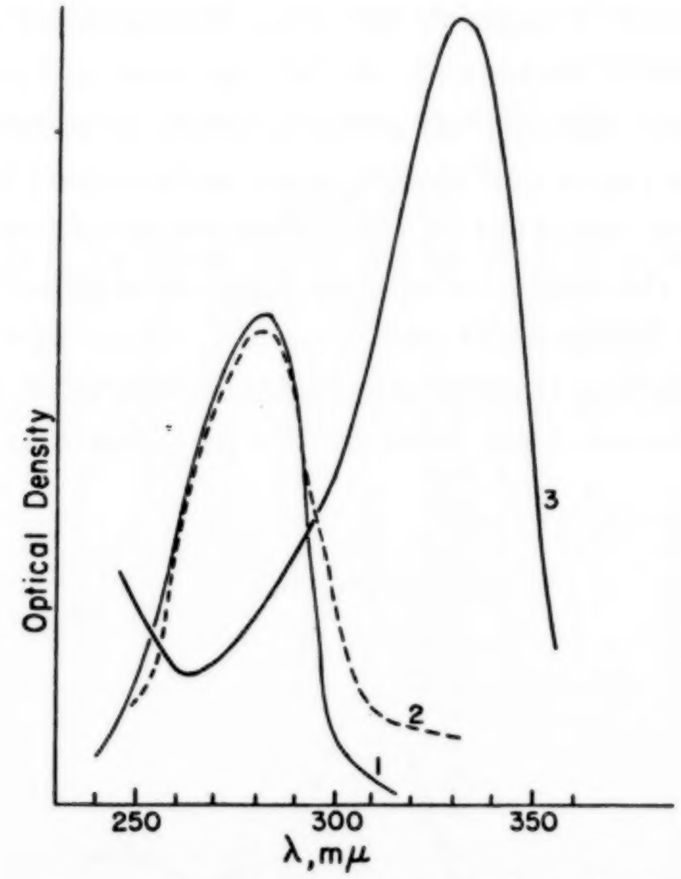


Fig. 1. Ultraviolet spectra of *p*-hydroxyacetophenone in *n*-butanol saturated with water: (1) alone; (2) with added Na_2HPO_4; (3) with added $NaBO_3$.

TABLE II

SPECTRA OF PHENOLS AFTER CHROMATOGRAPHY ON PLAIN PAPER, PHOSPHATE PAPER AND BORATE PAPER

Phenol	R_F			λ_{max} (mμ)		
	Plain	Na_2HPO_4	$NaBO_3$	*Plain*	Na_2HPO_4	$NaBO_3$
Phloroglucinol	0.78	0.66	0.27	268	273	283
p-Hydroxyacetophenone	0.97	0.96	0.82	279	325, 284	330
p-Hydroxybenzal-acetone	0.93	0.93	0.85	328	331	390
Apigenin	0.91	0.72	0.15	341, 269	380, 280	410, 335, 277
Apigenin 7-rhamnoglucoside	0.40	0.24	0.07	343, 269	352, 269	397, 268
Tricin	0.90	0.64	0.08	360, 270*	382, 277	428, 330, 272

* Inflection

and *o*-hydroxyacetophenone. It is clear, therefore, that sodium borate behaves like the strong base, sodium ethylate, and ionizes all of the hydroxyl groups in most phenols.

A number of the more highly conjugated phenols were finally chromatographed on plain paper, phosphate paper and borate paper as described in the chromatographic test. Lower R_F values were obtained on the borate paper in every case. After chromatography, the spectra of the migrated species were determined directly on the paper strips by the method of BRADFIELD AND FLOOD[12]. These spectra (Table II) closely agreed with the corresponding spectra in *n*-butanol solutions. Each of these phenols, therefore, exists in a different ionic form on the phosphate and borate paper. Thus, *p*-hydroxybenzal-acetone (Fig. 2) migrates as the un-ionized phenol (VII) on plain paper (R_F 0.93) and phosphate paper (R_F 0.93) and as the ionic species (VIII) on borate paper (R_F 0.85).

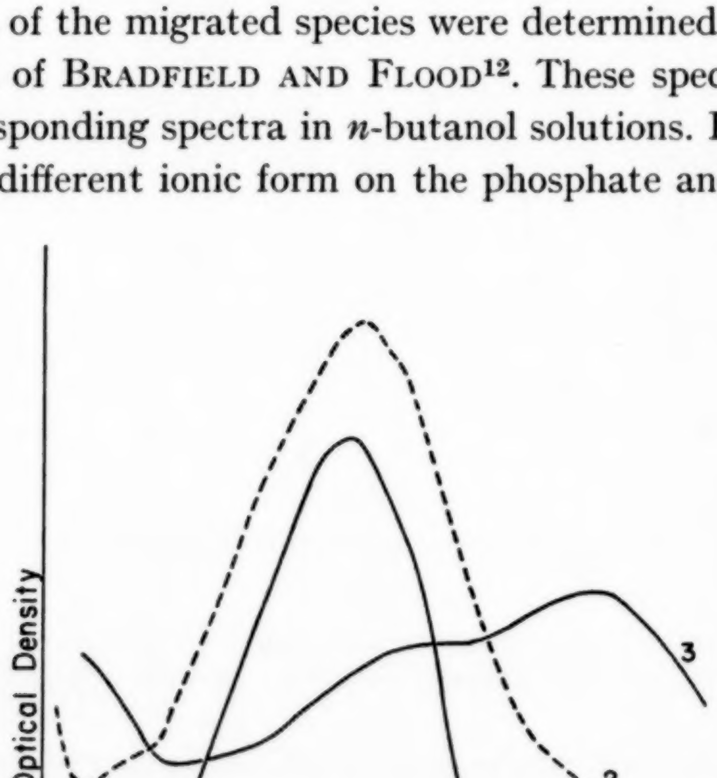
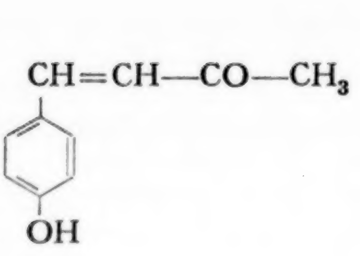


Fig. 2. Ultraviolet spectra (determined directly on the paper strip) of *p*-hydroxybenzal-acetone after chromatography on: (1) plain paper; (2) phosphate paper; (3) borate paper.



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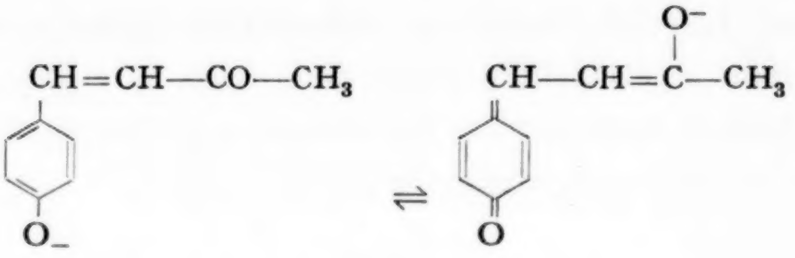

(VIII)

It has been reported[8] that in alcoholic solution a mixture of boric acid and sodium acetate reacts specifically with *ortho*-dihydroxyl groupings without ionization of other phenolic groups. A variety of phenols were therefore chromatographed on plain paper and on paper which had been impregnated with boric acid–sodium acetate. Phenols which lack an *ortho*-dihydroxyl grouping have approximately identical R_F values on both papers (Table III). Those which contain an *ortho*-dihydroxyl grouping have distinctly lower R_F values on the boric acid paper. This chromatographic procedure cannot be applied to free aromatic acids since the carboxyl group is ionized by the boric acid–sodium acetate reagent. It is especially useful,

TABLE III

R_F VALUES OF PHENOLS ON PLAIN PAPER AND ON BORIC ACID–SODIUM ACETATE PAPER

Phenol	*R_F in n-BuOH/H$_2$O*		*Difference**
	Plain	*H_3BO_3-NaOAc*	
Resorcinol	0.94	0.98	0.04
Hydroquinone	0.90	0.98	0.08
Phloroglucinol	0.78	0.83	0.05
Methyl *p*-coumarate	0.97	0.97	0
p-Hydroxybenzal-acetone	0.97	0.98	0.01
Naringenin	0.97	0.93	— 0.04
Hesperetin	0.94	0.93	— 0.01
Apigenin	0.95	0.91	— 0.04
Diosmetin	0.91	0.89	— 0.02
Rhoifolin	0.48	0.65	0.17
Naringin	0.55	0.66	0.11
Ombuin (7,4′-di-O-methylquercetin)	0.82	0.83	0.01
Catechol	0.93	0.66	— 0.27
Pyrogallol	0.78	0.29	— 0.49
l-Epicatechin	0.54	0.23	— 0.31
Methyl gallate	0.81	0.49	— 0.32
Methyl 3-O-methylgallate	0.87	0.67	— 0.20
Ethyl caffeate	0.89	0.75	— 0.14
Eriodictyol	0.97	0.74	— 0.23
Luteolin	0.95	0.48	— 0.47
Quercetin	0.78	0.48	— 0.30
Rhamnetin (7-O-methylquercetin)	0.82	0.64	— 0.18
Quercitrin	0.78	0.47	— 0.31
Taxifolin	0.82	0.58	— 0.24
3,3′,4′-Trihydroxyflavone	0.86	0.68	— 0.18
p-Coumaric acid	0.89	0.29	— 0.60
Ferulic acid	0.89	0.62	— 0.27
Caffeic acid	0.81	0.22	— 0.59
Gallic acid	0.62	0.13	— 0.49

* Difference = R_F on boric acid/sodium acetate paper — R_F on plain paper.

however, for differentiating *ortho*-dihydroxyflavanones which cannot be detected spectrophotometrically[9], compounds such as eriodictyol and taxifolin being easily distinguished from similar flavanones, *e.g.*, hesperetin and naringenin, which do not contain an *ortho*-dihydroxyl group.

EXPERIMENTAL

Ultraviolet spectra

All spectra were determined at room temperature on a Beckman model DU spectrophotometer with 1 cm silica cells.

The spectrum of each of the phenols listed in Table I was first determined in *n*-butanol saturated with water. Powdered disodium hydrogen phosphate or sodium borate was then added to saturate the solution and after 10 minutes, the spectrum was redetermined.

Chromatography on boric acid–sodium acetate paper

The boric acid–sodium acetate paper was prepared by steeping Whatman No. 1 paper in a saturated solution of sodium acetate in absolute ethanol for 10 minutes. The air-dried paper was then steeped in a saturated solution of boric acid in absolute ethanol for 10 minutes and air-dried.

The R_F values of the phenols listed in Table III were determined by simultaneous ascending chromatography on plain paper and the boric acid–sodium acetate paper, using *n*-butanol saturated with water as solvent.

Chromatography on phosphate and borate papers

Papers impregnated with 0.1 M Na_2HPO_4 and 0.1 M $NaBO_3$ were prepared as described by SWAIN[2]. The R_F values of the phenols listed in Table II were determined by ascending chromatography on these papers, using *n*-butanol saturated with water as solvent. The phenols were located on the developed chromatograms by their fluorescence in U.V. light. Their spectra were then determined on the dried paper strips by the method of BRADFIELD AND FLOOD[12].

SUMMARY

1. The influence of sodium phosphate (Na_2HPO_4) and sodium borate ($NaBO_3$) on the ionization of phenols has been determined spectrophotometrically. Na_2HPO_4 ionizes only strongly acidic phenolic hydroxyl groups whereas sodium borate ionizes most phenolic groups. Lower R_F values on borate impregnated paper, therefore, are not necessarily due to the presence of *ortho*-dihydroxyl groups but may arise from ionization of individual hydroxyl groups.

2. Phenolic hydroxyl groups are not ionized by a mixture of boric acid–sodium acetate. Phenols containing *ortho*-dihydroxyl groupings may be detected chromatographically by their lower R_F values on boric acid–sodium acetate impregnated paper as compared with plain paper.

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ULTRAVIOLET PHOTOLYSIS OF NUCLEOSIDES AND NUCLEOTIDES ON FILTER PAPER

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INTRODUCTION

The photolysis of nucleic acids and related compounds caused by ultraviolet irradiation is of considerable interest because of the important role that these compounds play in a variety of cellular functions and because of their high selective absorption in the ultraviolet region. Many publications in this field demonstrate the photosensitivity of pyrimidines, their nucleosides and nucleotides as compared to purines and their derivatives[1-4]. In most instances, the photolysis of purines, pyrimidines and their derivatives including nucleosides, nucleotides and nucleic acids is accompanied by the reversible destruction or complete loss of their absorption characteristics[1-6]. In addition to a variety of means of identifying the nucleic acid bases on paper chromatograms, a method was presented in a previous communication[7] in which the extreme instability of uracil and thymine towards ultraviolet light was used. The work described in this paper deals with further studies on the photosensitivity of pyrimidine nucleosides and nucleotides, in particular of the compounds that contain either uracil or thymine as a base component.

EXPERIMENTAL

Materials

The ribonucleosides were prepared from baker's yeast ribonucleic acid, while the ribonucleotides were of commercial grade, which were recrystallized before use. The deoxyribonucleosides were purchased from California Corporation for Biochemical Research, U.S.A., and were used as received. All these compounds were checked for impurities by chromatography and spectrophotometry.

In general, the samples were employed as aqueous solutions, but some preparations that are difficultly soluble in water were dissolved in suitable solvents. Thus, guanosine was dissolved in 1/200 M sodium hydroxide solution, adenylic and guanylic acid were used as solutions in 2/100 M citrate buffer of pH 3.0. The concentration of each solution was 15 mM. Spotting on filter paper (Toyo No. 51-A) was carried out so that each spot contained 0.15 μmole of the sample.

Ultraviolet irradiation

In most cases ultraviolet photolysis of the ribonucleosides and nucleotides spotted on filter paper was performed after chromatographic or electrophoretic separation. In the case of the deoxyribonucleosides, the irradiation was carried out with the compounds spotted separately on a paper sheet. Irradiation was performed as described before[7] by means of a commercial germicidal lamp (15 W, "National" of Matsushita Electric Ind. Co. Ltd., Osaka) at 30 mm distance without cut-off filter.

Detection

(a) The chromatograms were photographed with an ultraviolet source of the same type as employed in the irradiation technique, using a filter capable of isolating the 253.7 mμ emission line. This was kindly supplied by Dr. IWASE's Laboratory of the Institute for Scientific Research, Tokyo.

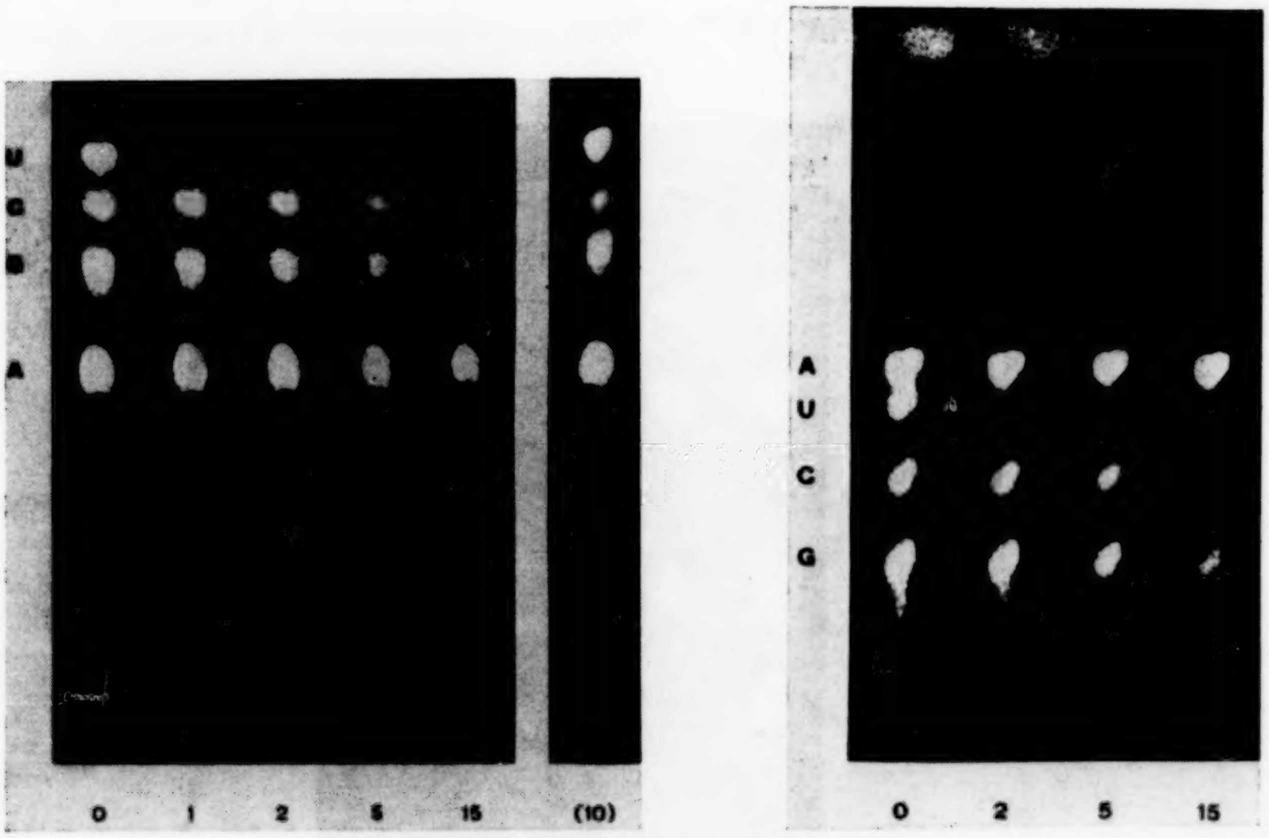
(b) Paper sheets submitted to photolysis were sprayed with acidic *p*-dimethylaminobenzaldehyde solution, which was employed for the detection of pyrimidine reduction products such as α-ureidopropionic acid, allantoin, urea, etc.[8]. The sheets were then hung in a ventilated room at room temperature for several hours.

(c) For the deoxyribonucleosides, the diphenylamine reaction was also employed in the usual manner[9].

RESULTS

Ultraviolet absorption

Ribonucleosides. From the accompanying photographs (Fig. 1) it can be seen that on exposure of the chromatogram to ultraviolet light only uridine lost its characteristic spot within 2 h of irradiation. Other nucleosides, whose spots became weaker



a

b

Fig. 1. Changes in ultraviolet prints of ribonucleosides on paper chromatograms as a function of irradiation period. Irradiation was carried out after a chromatographic run with (a) water (adjusted to pH 10 by aqueous ammonia)[10] or (b) *n*-butanol–isobutyric acid–25 % aqueous ammonia–water (75:37.5:2.5:25)[11]. The numbers indicate the irradiation period in hours without filter except (10) which stands for 10 h irradiation through a glass filter of 1 mm in thickness. A = adenosine, G = guanosine, C = cytidine, and U = uridine.

to a greater or lesser extent with increasing irradiation time, remained almost unaffected under the conditions applied. Prolonged photolysis caused the disappearance of cytidine followed by guanosine. However, when the spots containing uridine and other nucleoside or nucleosides were subjected to irradiation for 3 h and developed with the same solvent systems as employed in the above chromatographic run, uridine appeared on the chromatograms as a fairly distinct spot. In other words, long irradiation was necessary to eliminate the particular spot or spots when the mixed sample was photolysed only before the chromatographic run. Thus, uridine and cytidine were still found together with purine nucleosides on the chromatogram when the mixed sample spotted was irradiated for 10 h before development. If uridine alone was photolysed for 3 h and treated in the same way, the spot could hardly be found on the chromatogram. Since the irradiation period required to eliminate the spot is a function of the amount of the compound present per unit area, it goes without saying that longer irradiation is necessary when photolysis is carried out before development, because spots appearing after a chromatographic run are always larger than the original ones. Another paper strip, which was developed and treated in the same way, was irradiated through a glass plate of 1 mm thickness. No significant changes in the chromatogram were observed before and even after long irradiation, indicating the protective effect of the glass filter on the sensitivity of the compounds towards ultraviolet light. This was also the case with nucleotides and deoxyribonucleosides.

Ribonucleotides. Ribonucleotides showed a behavior similar to that of the corresponding nucleosides with regard to ultraviolet irradiation. The compounds were separated by electrophoresis at pH 3.7 [12]. A representative ultraviolet diagram is

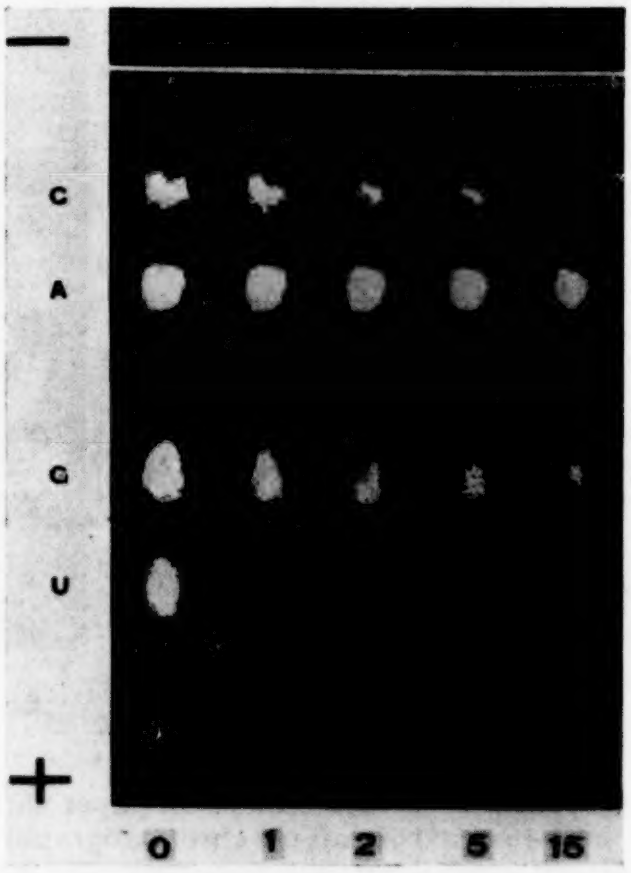


Fig. 2. Changes in ultraviolet prints of ribonucleotides after electrophoretic separation in an acetate buffer, ionic strength 0.030, pH 3.7 [12]. A = adenylic acid, G = guanylic acid, C = cytidylic acid, and U = uridylic acid.

shown in Fig. 2, which indicates that uridylic acid disappears after short irradiation.

Deoxyribonucleosides. A paper strip was spotted with each sample solution, the spots having a diameter of 7–9 mm, and then submitted to ultraviolet irradiation; photographic prints were made at intervals. As shown in Fig. 3, the appearance of the ultraviolet prints is similar to that expected, *viz.*, thymidine followed by deoxycytidine lose their absorption characteristics as the time of irradiation increases, while no major differences can be seen in the spots corresponding to the purine deoxyribosides. However, deoxyguanosine gave a faint spot, as in the case of guanosine and guanylic acid in Figs. 1 and 2, when irradiation was carried out for 15 h after the chromatographic run.

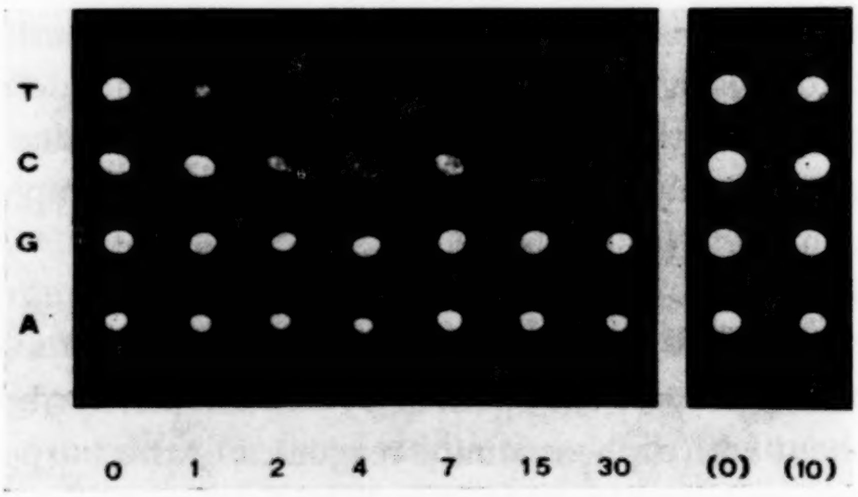


Fig. 3. Changes in ultraviolet prints of deoxyribonucleosides on a paper strip. Numbers in parentheses indicate the irradiation period through a glass plate as employed in Fig. 1. A = deoxyadenosine, G = deoxyguanosine, C = deoxycytidine, and T = thymidine.

Coloration with acidic p-*dimethylaminobenzaldehyde*

In the photolysis of thymine and uracil on filter paper, no urea formation was observed contrary to the finding of BASS[13] that photolysis of thymine in aqueous solution results in the formation of urea as one of the main products. We found, however, that after photolysis some compounds gave a coloration with acidic *p*-dimethylaminobenzal-

TABLE I

TIME REQUIRED TO PRODUCE YELLOW SPOTS AFTER SPRAYING WITH ACIDIC *p*-DIMETHYLAMINOBENZALDEHYDE SOLUTION AS A FUNCTION OF IRRADIATION PERIOD

Base or base component	*Irradiation period*					
	30 min	*1 h*	*2 h*	*5 h*	*10 h*	*20 h*
Adenine	—	—	—	+	> 6	4 ~ 6
Guanine	—	—	+	> 6	3	1
Cytosine	—	—	+	2 ~ 4	1 ~ 2	½ ~ 1
Uracil	+	5 ~ 6	1 ~ 2	½ ~ 1	½ >	½ >
Thymine	1	½	½	½ >	½ >	½ >

The numbers indicate the time in hours required for the spots to become distinctly visible after spraying the reagent. Nucleosides and nucleotides were expressed as their component bases since no significant differences were observed between the base and the derivatives.

+ = faint coloration after 10 h.
— = no coloration after 24 h.

dehyde, which reagent produces a yellow spot when sprayed on compounds with the general formula RHN—CO—NH_2. 0.01 ml aliquots of each sample solution were spotted on filter paper, so that the diameter of the spots was 15–18 mm, and then submitted to irradiation for various periods. After spraying the reagent, the paper was hung in a ventilated room in order to determine the time required for the appearance of distinctly visible spots. In Table I the results obtained with the nucleosides and nucleotides as well as with their component bases are listed. It can be seen that 5 h photolysis is suitable for the performance of the color reaction. The sensitivity (tentatively defined as the minimum amount of the compound submitted to 5 h photolysis which produces a distinctly visible spot within 6 h after spraying the reagent), differs for the various bases or basic components in nucleosides and nucleotides. Thus, the sensitivity is 0.2 µmole for guanine and its derivatives, 0.15 µmole for cytosine and its derivatives, 0.1 µmole for uridine and its derivatives and 0.04~0.06 µmole for thymine and thymidine, respectively. With adenine and its derivatives, color production was scarcely possible under the conditions employed even with 0.5 µmole of the compounds.

Coloration of deoxyribonucleosides with diphenylamine reagent

An attempt was made to reveal the spots of pyrimidine deoxyribosides by coloration of the sugar component with diphenylamine reagent. For this purpose, four nucleosides were spotted in a row on a paper sheet at a distance of 25 mm from each other, and subjected to photolysis for varying periods. The paper was then sprayed with diphenylamine reagent and heated at 90° for 10 min, the paper being clamped between two glass plates to avoid charring. It is well known that under the conditions employed here, only purine deoxyribosides give a coloration, because in pyrimidine nucleosides the glycosidic linkage is resistant towards acid hydrolysis. Fig. 4, which gives typical results, shows that pyrimidine nucleosides could also be detected together with purine deoxyribosides when photolysis for 2 h preceded the performance of the Dische reaction. It can also be seen that prior photolysis is more effective for this

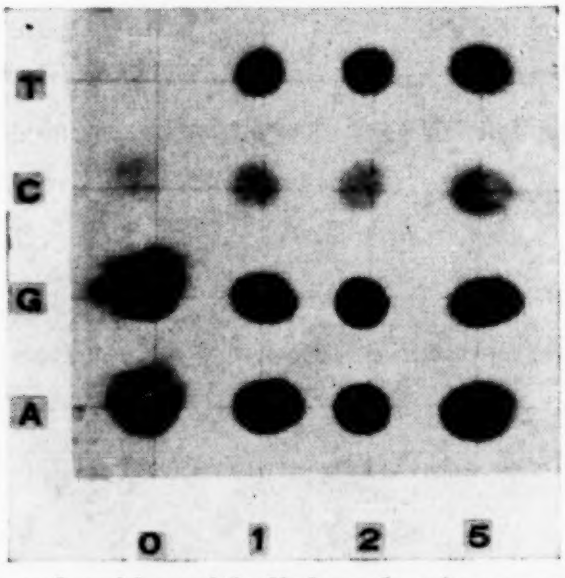


Fig. 4. Coloration of deoxyribonucleosides with diphenylamine reagent before and after photolysis for various periods.

purpose in the case of thymidine than in that of deoxycytidine. The diagram made after prolonged photolysis (24 h) showed features similar to those obtained after photolysis for 5 h. The sensitivity of thymidine was shown to be of the same order as that obtained in the bromination technique (0.06 μmole)[14].

DISCUSSION

The results presented in this paper and the previous one[7] clearly show the photosensitivity of pyrimidines, their nucleosides and nucleotides, particularly uracil, thymine and their derivatives, on the paper chromatograms. On the other hand, purines, purine nucleosides and nucleotides are fairly resistant towards ultraviolet light although guanine derivatives show some photodegradation after prolonged irradiation. Under the conditions employed, the photosensitivity of the compounds depends to a high degree on the species of the bases present and not on the presence or absence of the sugar or sugar-phosphate moiety. According to SHUGAR AND WIERZCHOWSKI[15], pyrimidines and their derivatives including nucleosides and nucleotides may be divided into two groups from the photochemical point of view. Thus, the photolysis of compounds without a substituent at position 4 or 5 of the pyrimidine ring is to a greater or lesser degree reversible; here uptake of a water molecule by the 4,5-double bond is concerned. On the other hand derivatives such as thymine, which are substituted at position 4 or 5 suffer destruction of the pyrimidine ring to give a variety of products. Hence the changes are no longer reversible. We have examined these interesting phenomena with the same source employed in this study and have obtained results similar to those of SINSHEIMER AND HASTINGS[1], SINSHEIMER[16], and SHUGAR AND WIERZCHOWSKI[17], *viz.* that the principal absorption maxima of uridylic acid, uracil and cytosine disappeared or were diminished by the ultraviolet irradiation at 253.7 mμ, but regained their former values, either partially or nearly completely, on acidifying the solution to pH1,[1,16] or by heating the solution at neutral pH[1,17]. It must be mentioned here that the investigations concerning the reversible photolysis of pyrimidine derivatives were carried out either in an aqueous or a buffered solution in many instances, whereas in the present study the photolysis of the compounds was performed on filter paper. This latter method might be regarded as photolysis in the dry state in the presence of air. Under the conditions employed, photolysis of all the compounds examined seems to be almost irreversible, since the eliminated spots did not reappear after development even with strong acidic solvents such as isopropanol–hydrochloric acid[18]. Restoration by visible light, which was demonstrated in the case of diphosphopyridine nucleotide[19] was also not observed in this study.

The mechanism of ultraviolet photolysis as investigated in the present study has not yet been well understood; however, cleavage of the pyrimidine ring may play a leading role in diminishing or destroying the ultraviolet absorptivity of the compounds, since even after 30 min photolysis, thymine and thymidine and after longer photolysis, other pyrimidines as well as their nucleosides and nucleotides react with *p*-dimethylaminobenzaldehyde reagent to form yellow spots. Cleavage of the purine ring in guanosine and in guanylic acid is also suggested. Whatever the nature of the degradation products may be, whose elucidation will be the subject of a subsequent paper, the method of spraying with *p*-dimethylaminobenzaldehyde may be of some value when analysing the bases, nucleosides and nucleotides after prior photolysis of the compounds.

Furthermore, these ultraviolet irradiation techniques have also further value in some special cases where the photodegradation of pyridine deoxyribosides is concerned. A method of detecting deoxyribosides and ribotides on paper chromatograms based on the color reactions of their sugar component, *e.g.*, the cysteine–sulphuric acid reaction[20], has been used together with the ultraviolet absorption technique. Diphenylamine spray[9], generally employed for the detection of purine derivatives was found to be applicable also for the detection of pyrimidine derivatives when the compounds were brominated before performance of the Dische reaction[14]. The technique presented here may also be effective for this purpose, in particular for the detection of thymidine.

Although the photolysis was not carried out under strict conditions, particularly without cutting off the emission lines at wavelengths below 230 mμ, it might be of value as an auxiliary method for the detection and identification of the compounds because of its simplicity and the applicability to a variety of compounds.

ACKNOWLEDGEMENTS

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SUMMARY

Nucleosides and nucleotides were irradiated on filter paper with ultraviolet light. Pyrimidine nucleosides and nucleotides, in particular compounds containing uracil or thymine as a base component were extremely unstable towards ultraviolet irradiation. After photolysis, pyrimidine nucleosides and nucleotides lost their absorption characteristics and produced a yellow color when sprayed with acidic *p*-dimethylaminobenzaldehyde solution. The slow degradation of guanine derivatives was also demonstrated whereas adenine-containing compounds were the most resistant. Pyrimidine deoxyribosides reacted with Dische's reagent after photolysis. These observations may be applicable for the detection and identification of the compounds on chromatograms.

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ANWENDUNG DER DÜNNSCHICHTCHROMATOGRAPHIE AUF PTERIDINE

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(Eingegangen den 15. März 1960)

Die papierchromatographische Analyse wurde auf dem Gebiet der synthetischen[1-7] sowie der natürlichen Pteridine[8-12] bereits angewendet, wobei sich TSCHESCHE UND KORTE[1-2] durch eine systematische Untersuchung der Verwendbarkeit von verschiedenen Fliessmitteln insbesondere hervortaten. Im Verlauf früherer synthetischer Arbeiten auf dem Pteridingebiet[13-15] haben wir wiederholt Gelegenheit gehabt die Papierchromatographie anzuwenden, wobei uns öfters auffiel, wie manche einheitliche Pteridine mit gewissen Fliessmitteln zur Schwanzbildung neigen oder sogar mehrere Flecken gleicher Fluoreszenzfarbe ergeben. Die Neigung zur Schwanzbildung scheint in engem Zusammenhang mit einem Gleichgewicht zwischen ionisierter und nicht-ionisierter Form zu stehen[16], während die Bildung mehrerer Flecken bei reinen Verbindungen mit der Entstehung von hydratisierten Formen (Addition von Wasser an einer Doppelbindung)[7] oder einer Keto-Enol-Tautomerie[17] erklärt werden dürfte.

In den letzten Jahren wurde die Chromatographie an Aluminiumoxyd und Silicagel soweit ausgebaut, dass es heute möglich ist, geringe Mengen von Substanzgemischen in die einzelnen Komponenten zu trennen. Zu diesem Zweck verwendete als erster FLOOD mit Aluminiumoxyd imprägnierte Filtrierpapierstreifen zur Trennung von anorganischen Ionen[18]. Diese Methode wurde daraufhin auch in der organischen Analyse ausgebaut, wobei neben Aluminiumoxyd auch Silicagel verwendet wurde[19-22]. Vom imprägnierten Papierstreifen wurde dazu übergangen, das Adsorptionsmittel mit einem Bindemittel vermischt, in dünner Schicht auf Glasstreifen oder Platten aufzuziehen, wobei STAHL die letztere Methode besonders eingehend ausbaute[23-27]. Als Bindemittel wurden Stärkekleister[28-31] und Gips[23-28, 32] vorgeschlagen.

Bei Wiederaufnahme unserer Arbeiten auf dem Pteridingebiet versuchten wir eine schnelle analytische Methode auszuarbeiten, welche es gestattet mit kleinen Mengen sichere Angaben über die Reinheit der geprüften Stoffe zu gewinnen und etwaige Stoffgemische in die Komponenten zu spalten.

Zu diesem Zweck wendeten wir die sogenannte "Dünnschicht-Chromatographie" nach STAHL[23-27] mit Erfolg an. Sie erlaubte uns nämlich Trennungen, welche bisher im Papierchromatogramm misslungen waren. Als Adsorptionsmittel verwendeten wir

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* Neue Anschrift: bei Lepetit S.p.A., Reparto Ricerche, Milano (Italien).

J. Chromatog., 4 (1960) 384-390

Kieselgel[24]* oder Aluminiumoxyd** mit Gips vermischt. Die verwendeten Fliessmittel sind in Tabelle I zusammengestellt. Die Herstellung und Aktivierung der Platten wird im EXPERIMENTELLEN TEIL beschrieben. Beispiele von Trennungen werden in den Tabellen II–IV und Fig. 1 und 2 erläutert. Die Unterscheidung der Substanzen gründet sich nicht nur auf den R_F-Wert. Die zum Teil charakteristischen und verschiedenen Fluoreszenzfarben gestatten es, Stoffe mit praktisch beieinander liegenden Laufstrecken zu unterscheiden. Wegen den relativ grossen Fehlerbreiten der letzteren wäre es empfehlenswert, dieselben auf eine Standardsubstanz zu beziehen.

Die verwendeten Fliessmittel lassen sich in drei Klassen einteilen: neutrale, saure und alkalische.

TABELLE I

LAUFMITTEL ZUR ANALYTISCHEN TRENNUNG VON PTERIDIN-DERIVATEN

Nr.	*Zusammensetzung der Laufmittel*
1	190 ml Dimethylformamid/10 ml Wasser
2	20 ml Dimethylformamid/180 ml Wasser
3	400 ml Benzol feucht/80 ml Dimethylformamid
4	400 ml *n*-Butylalkohol/400 ml Wasser/100 ml Pyridin
5	400 ml *n*-Butylalkohol mit Wasser gesättigt/80 ml Dimethylformamid
6	100 ml Dimethylformamid/100 ml Wasser
7	180 ml Dimethylformamid/20 ml Wasser
8	198 ml Dimethylformamid/2 ml Wasser
9	Dimethylformamid 100 %
10	200 ml *n*-Butylalkohol/100 ml Essigsäure 5*N*
11	200 ml Isopropylalkohol/100 ml Ammoniak 1 %
12	Citronensäure 5 %
13	Ammoniumchlorid 5 %
14	Citronensäure 1 %
15	Citronensäure 10 %
16	Äpfelsäure 5 %
17	Weinsäure 5 %
18	Milchsäure 5 %
19	Ammoniak 5 %
20	Ammoniak 10 %
21	Ammoniak 1 %
22	100 ml Ammoniak 5 %/5 g Ammoniumchlorid
23	100 ml Ammoniak 5 %/100 ml Isopropylalkohol
24	Ammoniak 25 %

Von den verwendeten neutralen Dimethylformamid–Wasser Gemischen ergeben Nr. 1 und 2 allgemein bessere Resultate als diejenigen mit höherem oder niedrigerem Wasser-Gehalt (Nr. 3, 5–9). Mit Fliessmittel 1 und 2 wandern in Stellung 6 und/oder 7 hydroxylierte und/oder carboxylierte Pteridine nicht, im Gegensatz zu den unsubstituierten oder durch Methylgruppen substituierten Abkömmlingen.

Von den sauren Fliessmitteln ergibt Nr. 10 nur mit 2,4-Dihydroxypteridin (Lumazin) und 6- bzw. 7-Methylpteridin schärfere Zonen. Ammoniumchlorid-Lösung ergibt durchwegs sehr unscharfe Trennungen. 1-, 5- und 10 %ige Citronensäure, sowie

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* Kieselgel G nach STAHL für Dünnschichtchromatographie (Firma Merck A. G. Darmstadt).

** Aluminiumoxyd nach TSCHERTER für Dünnschichtchromatographie (Firma Fluka A.G. Buchs/St. Gallen).

TABELLE II

BEISPIELE DER ANALYTISCHEN TRENNUNG VON PTERIDIN-DERIVATEN AUF KIESELGELPLATTE

Nr.	*Substanz*	*R_F-Werte in verschiedenen Fliessmitteln (26°)*					
		1	*12*	*14*	*15*	*16*	*17*
4	2-Amino-4-hydroxypteridin	0.55 ± 0.05	0.78 ± 0.05	0.80 ± 0.05	0.72 ± 0.05	0.82 ± 0.05	0.72 ± 0.05
5	2-Amino-4-hydroxy-6,7-dimethylpteridin	0.65 ± 0.1	0.46 ± 0.02	unscharf	0.61 ± 0.05	unscharf	unscharf
6	2-Amino-4-hydroxy-6-methylpteridin	0.65 ± 0.05	0.69 ± 0.02	0.66 ± 0.05	0.75 ± 0.05	0.62 ± 0.05	unscharf
7	2-Amino-4-hydroxy-7-methylpteridin	0.88 ± 0.05	0.60 ± 0.01	0.68 ± 0.05	0.68 ± 0.05	0.64 ± 0.05	0.58 ± 0.05
12	2,4-Dihydroxypteridin (Lumazin)	0.0	0.88 ± 0.05	0.95 ± 0.05	0.89 ± 0.05	unscharf	0.83 ± 0.05

TABELLE III

BEISPIELE DER ANALYTISCHEN TRENNUNG VON PTERIDIN-DERIVATEN AUF KIESELGELPLATTE

Nr.	*Substanz*	*R_F-Werte in verschiedenen Fliessmitteln (26°)*					
		19	*20*	*21*	*22*	*23*	*24*
1	Leucopterin[33]	unscharf	unscharf	unscharf	unscharf	0.0	0.0
2	6-Methylisoxanthopterin*[34]	unscharf	0.85 ± 0.05	unscharf	unscharf	unscharf	0.88 ± 0.05
3	7-Methylxanthopterin*[34]	unscharf	0.83 ± 0.06	unscharf	0.82 ± 0.05	0.55 ± 0.03	0.88 ± 0.05
4	2-Amino-4-hydroxypteridin[35]	0.92 ± 0.05	0.95 ± 0.05	unscharf	0.85 ± 0.05	0.80 ± 0.02	0.92 ± 0.02
5	2-Amino-4-hydroxy-6,7-dimethylpteridin[35]	0.88 ± 0.05	0.91 ± 0.05	unscharf	*ca.* 0.75	0.81 ± 0.03	0.91 ± 0.02
6	2-Amino-4-hydroxy-6-methylpteridin[36]	0.79 ± 0.05	0.93 ± 0.05	unscharf	0.88 ± 0.02	0.83 ± 0.02	0.92 ± 0.02
7	2-Amino-4-hydroxy-7-methylpteridin[36, 37]	0.92 ± 0.05	0.92 ± 0.06	unscharf	0.85 ± 0.02	0.86 ± 0.02	0.91 ± 0.02
8	Isoxanthopterin[38]	unscharf	unscharf	unscharf	unscharf	unscharf	0.8 ± 0.1
9	Xanthopterincarbonsäure*[38]	0.12 ± 0.05	0.11 ± 0.02	unscharf	0.07 ± 0.02	unscharf	0.07 ± 0.02
10	2-Amino-4-hydroxy-7-carboxypteridin[37]	unscharf	unscharf	unscharf	unscharf	unscharf	unscharf
11	2-Amino-4-hydroxy-6,7-dicarboxypteridin[39]	0.16 ± 0.05	0.12 ± 0.02	unscharf	*ca.* 0.18	0.0	0.10 ± 0.05
12	Lumazin[40]	0.88 ± 0.05	0.92 ± 0.01	unscharf	0.89 ± 0.03	0.81 ± 0.03	0.83 ± 0.03

* R_F-Wert der Hauptzone.

Äpfelsäure, Weinsäure und Milchsäure sind im Trenneffekt praktisch gleichwertig. Die Laufstrecken mit den sauren Fliessmitteln sind gut bei den in Stellung 7 durch Wasserstoff oder Methylgruppen substituierten Pteridinen, während die 7-Hydroxy- und 7-Carboxy-pteridine am Startort zurückbleiben. Lumazin wandert sehr gut.

Die Fliessmittel 4 und 11 ergeben nur mit Lumazin scharfe Trennungen. Mit den ammoniakhaltigen Laufmitteln wandern praktisch alle geprüften Pteridine. 1%iges Ammoniak ergibt unscharfe Zonen, während 5-, 10- und 25 %iges Ammoniak ähnliche Resultate liefern, wobei beim 10 %igem Ammoniak die Zonen im Durchschnitt etwas schärfer ausgebildet sind. Zusatz von Ammoniumchlorid zum 10 %igem Ammoniak verkürzt die Laufstrecke durchwegs, während Zugabe von Isopropylalkohol sich zum Teil durch Schwanzbildung ungünstig auswirkt.

Zusammenfassend kann man sagen, dass zur Trennung von 2-Amino-4-hydroxypteridine neutralen Charakters auf Kieselgelplatten saure, neutrale oder alkalische Fliessmittel geeignet sind, wobei den sauren, wegen den weiter auseinanderliegenden Laufstrecken, der Vorzug zu geben ist. Durch saure Gruppen substituierte 2-Amino-4-hydroxypteridine lassen sich mit alkalischen Laufmitteln besser trennen. Lumazin wandert sehr leicht mit neutralen, sauren und alkalischen Fliessmitteln. Die Laufstrecken auf Aluminiumoxydplatten sind erwartungsgemäss viel kleiner und in vielen Fällen gleich 0. Sämtliche geprüften Pteridine sind bereits bekannt und wurden nach den in Tabelle III zitierten Literaturangaben hergestellt. Sie wurden, wie vorgeschrieben, durch mehrmalige Umfällungen gereinigt bis die U.V.-Spektren den Angaben der Literatur entsprachen und die Elementaranalyse stimmte.

6-Methylisoxanthopterin (Nr. 2, Tabelle III), 7-Methylxanthopterin (Nr. 3, Tabelle III) und Xanthopterincarbonsäure (Nr. 9, Tabelle III), welche bis heute als rein galten, erwiesen sich im Dünnschichtchromatogramm als uneinheitlich, indem sie von kleinen Mengen von Stoffen mit anderer Fluoreszenzfarbe begleitet sind (vgl. Fig. 1).

TABELLE IV

BEISPIELE DER TRENNUNG VON LUMAZIN AUF KIESELGELPLATTE

R_F-Werte in verschiedenen Fliessmitteln (26°)

Fliessmittel Nr.	R_F	*Fliessmittel Nr.*	R_F
1	0.0	14	0.95 ± 0.02
2	0.95 ± 0.01	15	0.89 ± 0.03
4	0.57 ± 0.05	16	unscharf
5	0.63 ± 0.04	17	0.83 ± 0.05
9	0.0	19	0.88 ± 0.06
10	0.60 ± 0.05	20	0.92 ± 0.01
11	0.61 ± 0.05	21	unscharf
12	0.88 ± 0.05	22	0.89 ± 0.02
13	unscharf	23	0.81 ± 0.02
		24	0.83 ± 0.02

Die Verschiedenheit der Fluoreszenzfarbe der Begleitstoffe legt die Vermutung nahe, es handle sich um richtige Verunreinigungen, welche nach den üblichen Methoden nicht nachweisbar sind und nicht um einfache Entmischungen wie auf Seite 384 erwähnt.

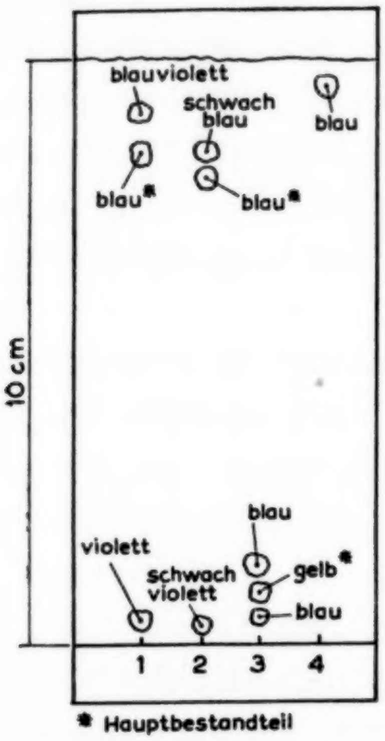

Fig. 1. Beispiel der Trennung von vier Pteridin-Derivaten auf Kieselgel-G-Platte. System: 10 %iges Ammoniak. Laufzeit: 20 min. (1) 1 γ 6-Methylisoxanthopterin; (2) 1 γ 7-Methylxanthopterin; (3) 1 γ Xanthopterincarbonsäure; (4) 1 γ 2-Amino-4-hydroxypteridin.

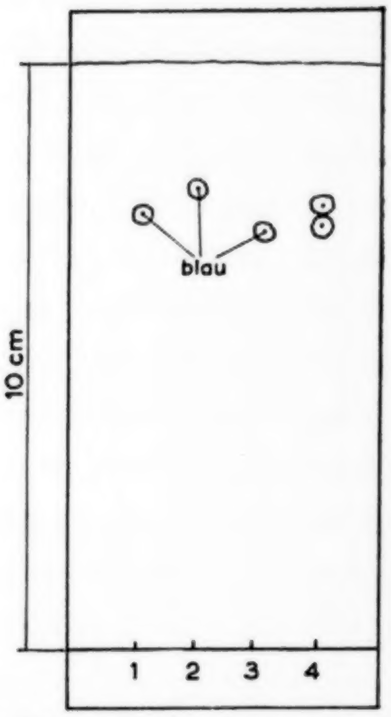

Fig. 2. Beispiel der Trennung von vier Pteridin-Derivaten auf Kieselgel-G-Platte. System: Citronensäure 10 %. Laufzeit: 30 min. (1) 1 γ 2-Amino-4-hydroxypteridin; (2) 1 γ 2-Amino-4-hydroxy-6-methylpteridin; (3) 1 γ 2-Amino-4-hydroxy-7-methylpteridin; (4) 2 γ Gemisch der Nr. 2 + 3.

Im Papierchromatogramm sind hingegen keine Entmischungen feststellbar. Die rohen 6- und 7-Methylpteridine[36, 37] sind Gemische beider Isomere. Nach Reinigung über die Acetylderivate sind die Produkte auch im Dünnschichtchromatogramm einheitlich.

EXPERIMENTELLER TEIL*

(*a*) *Kieselgelplatten*

25 g Kieselgel G nach STAHL (Merck) wurden in einem Erlenmeyerkolben von 200 ml Inhalt mit 50 ml destilliertem Wasser versetzt und 30 sec kräftig geschüttelt. Da die Mischung sehr rasch abbindet, giesst man sie sofort ins Streichgerät (Firma Desaga) und verstreicht die Platten in einem Zug. Als Trägerplatten dienten solche aus 5 mm dickem Pyrex-Glas, 10 × 20 cm. Hierauf wurden die Platten 4 St. bei 140° in einem Ofen getrocknet und durch Liegenlassen auf dem Tisch abkühlen gelassen und bis zur Verwendung verschlossen aufbewahrt.

Die Pteridine wurden als 1 %ige Lösungen in verdünntem Ammoniak, 2 cm vom unteren Rand in Abständen von 2 cm aufgetragen (Mengen 1–10 γ), hierauf die Platten in einem Ofen kurz getrocknet und in einen Glasturm mit dem Fliessmittel gestellt.

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[*] Die Angaben zur Herstellung der Platten verdanken wir Herrn HANS TSCHERTER (Fa. Sandoz A.G., Basel), dem wir auch an dieser Stelle unseren herzlichen Dank aussprechen möchten.

Die Platten sollen *ca.* 5 mm eintauchen. Nach Zurücklegen einer Strecke von 10 cm wurden die Platten herausgenommen, bei *ca.* 100° kurz getrocknet und im U.V.-Licht untersucht. Aufgetragen wurden 1–10 γ Substanz. 1 γ war in allen Fällen noch sehr gut sichtbar. Die Laufdauer betrug im Durchschnitt *ca.* 20 min.

(*b*) *Aluminiumoxydplatten*

15 g Aluminiumoxyd nach TSCHERTER (Fluka) wurden in einem Erlenmeyerkolben von 200 ml Inhalt mit 50 ml dest. Wasser versetzt und 30 sec kräftig geschüttelt. Weitere Arbeitsweise wie unter (*a*).

ZUSAMMENFASSUNG

Die Dünnschichtchromatographie nach STAHL wurde zur analytischen Trennung von Pteridinen erfolgreich angewendet.

SUMMARY

Thin layer chromatography according to STAHL was successfully applied to the separation of pteridines.

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PAPER CHROMATOGRAPHIC DATA ON THE PRODUCTS OBTAINED FROM ACID-TREATED SEDOHEPTULOSAN

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Ketoheptoses, especially sedoheptulose, have received increased attention recently because of their apparent role in carbohydrate metabolism reactions, and in a relatively short period of time a considerable amount of data, acquired in part by chromatographic techniques, have appeared in the literature. For purposes of comparison, sedoheptulose has been applied to the paper chromatogram in either the form of a sirup ordinarily obtained from the *Sedum* species or in the form of an "equilibrium" mixture obtained from the reaction of a dilute mineral acid on the crystalline anhydride, sedoheptulosan (2,7-anhydro-β-D-altro-heptulopyranose). The equilibrium mixture of the anhydride and the free sugar has been prepared by means of a mineral acid either at room temperature or at elevated temperatures, or by substituting the hydrogen form of an ion-exchange resin for the acid. With one possible exception, sedoheptulose has never been separated in its crystalline form[1,2]. In some instances, published chromatographic data on the components obtained by acid treatment of sedoheptulosan have been seemingly inconsistent and perhaps confusing, primarily because different investigators have used varied procedures in obtaining the data, *i.e.*, different methods of preparing the equilibrium mixture, different irrigants in preparing the chromatograms, variations in the application of the chromogenic reagent, and unstandardized times of heating the stained chromatograms.

KLEVSTRAND AND NORDAL[3] first showed that sedoheptulose and mannoheptulose would produce, on heating the paper chromatograms, a specific color reaction with an acidic solution of orcinol. This was later confirmed for all of the known heptuloses[4,5]. Several discrepancies appeared in this early work in regard to the chromatographic data on the anhydride, sedoheptulosan. Depending on the conditions used, sedoheptulosan would give either a positive or a negative response to orcinol treatment on the paper chromatograms. ZILL AND TOLBERT[6] suggested that this discrepancy was caused by the differences in time of heating the chromatograms. Variable data have also been reported on the components obtained by acid treatment of the anhydride. These differences were no doubt caused by variations in acid concentration, time of reaction, and temperature. UJEJSKI[2] reported that sedoheptulosan treated with 3 N hydrochloric acid for 12 hours at room temperature produced two spots on the chromatogram. DISCHE[7] observed three spots on the chromatogram when the anhydride was treated with 1% hydrochloric acid on a steam bath for 30 minutes. NOGGLE[5] observed four spots when the anhydride was treated with 0.2 *N* hydrochloric acid in a boiling water bath for one hour. Replacing the acid with the hydrogen form of the ion-exchange resin Dowex-50, NOGGLE[5] observed only two components on the chromatogram. However, ZILL AND TOLBERT[6], also using Dowex-50 followed by fractionation of the products on a Dowex-1 resin column, reported the isolation of four components. Subsequent investigations confirmed the possibility of having as many as four components in the mixture after treatment of the sedoheptulosan with acid; the amount of each component formed was dependent upon the conditions of acid concentration, temperature, and time[1]. These four components were (I) sedoheptulose (D-altro-heptulose); (II) sedoheptulosan (2,7-anhydro-β-D-altro-heptulopyranose); (III) 2,7-anhydro-β-D-altro-heptulofuranose; and (IV) tentatively identified as 5-(1,2-dihydroxyethyl)-2-furaldehyde[1].

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Because of our primary interest in obtaining reproducible and informative data on the heptuloses by paper chromatography, we undertook additional experiments to test the reaction of the orcinol reagent with the various components of the acid-treated sedoheptulosan. It is hoped that the data given below will clarify the discrepancies previously noted in the literature.

Using conditions of time, temperature, and acid concentration similar to those described by RICHTMYER AND PRATT[1] with the modification of employing 5% solutions of sedoheptulosan, we prepared mixtures in sealed glass tubes (Table I). After treatment, each mixture was stored at 0° until all were available for chromatography. Aliquots of 3 µl and 6 µl were applied to the chromatograms. Similar amounts of mannoheptulose, sedoheptulose (*Sedum* sirup), and untreated sedoheptulosan were also applied to the chromatograms as authentic standards. Data were obtained with different irrigants and chromatographic papers (see Table II). All chromatograms were prepared at room temperature, 25°.

TABLE I

CONDITIONS USED FOR ACID-TREATMENT OF SEDOHEPTULOSAN

Sample	*Heating time (h)*	*Temp. °C*	*Normality HCl*
1	140	40	0.2
2	40	60	0.2
3	4	80	0.2
4	125	80	0.2
5	1	100	0.2
6	50	100	0.2
7	240	25	0.2
8	240	25	0.5
9	240	25	1.0

The air-dried chromatograms were dipped twice in the orcinol solution (0.5 g orcinol, 15.0 g trichloroacetic acid, 100 ml isopropanol), and the sheets were air-dried

TABLE II

PAPER CHROMATOGRAPHIC DATA ON ACID-TREATED SEDOHEPTULOSAN

Irrigants	*Chromatographic paper**	*Distance migrated on chromatogram (mm)*						
		Standards			*Products from acid-treated sedoheptulosan***			
		Manno-heptulose	*Sedohep-tulosan*	*Sedohep-tulose*	*I*	*II*	*III*	*IV*
Ethyl acetate–pyridine–water (8:2:1) 15–16 h	E&D 248	53	66	88	81	68	123	
	What 1	57	60	80	80	65	115	
	E&D 048	67	80	108	106	88	154	
	E&D 613	76	81	114	109	90	157	
	E&D 5	76	98	124	125	103	178	
	E&D 611	104	114	149	150	119	210	
	E&D 4	159	185	225	227	192	314	
	E&D 7	183	188	239	238	200	327	
n-Butanol–propionic acid–water*** 30 h	What 1	65	92	68	64	85	117	287
n-Butanol–ethanol–water (10:1:2) 68 h	What 1	108	124	128	126	124	187	

* E&D = Eaton-Dikeman Company, Mt. Holly Springs, Pennsylvania. What = Whatman, H. Reeve Angel & Company, Inc., Clifton, New Jersey.

** I = sedoheptulose; II = sedoheptulosan; III = 2,7-anhydro-β-D-altro-heptulofuranose; IV = 5-(1,2-dihydroxyethyl)-2-furaldehyde (tentative identification).

*** Soln. A: 1246 ml *n*-butanol and 84 ml water. Soln. B: 620 ml propionic acid and 790 ml water. Equal volumes of A and B are mixed and adjusted to give a single-phase solution which becomes cloudy upon cooling to 22° or 2° below the thermostated room temperature[8].

after each dipping. The orcinol-treated sheets were heated in a forced-air oven at 105° for one minute. The sheets were removed from the oven, the spots formed within one minute were suitably marked, and the sheets were returned to the oven and heated for 30 minutes at 105°. Within one minute of heating, sedoheptulose appeared on the chromatograms. Within 30 minutes of heating, sedoheptulosan (II) and component (III) appeared. (Although the prescribed periods for heating the chromatograms will not be exactly reproducible in all ovens maintained at 105°, there will always be a marked difference in time required to develop the orcinol-treated sedoheptulose spots and the time required for developing the spots of the two anhydrides, (II) and (III).) With orcinol, fast-moving component (IV) produced a brilliant pink spot on the chromatogram immediately (prior to heat treatment), which changed to the characteristic blue color of the heptuloses with the application of heat. This component (IV) is most probably 5-(1,2-dihydroxyethyl)-2-furaldehyde[1,6]. The time-sequence of the appearance of the spots on the chromatograms confirms the observations of ZILL AND TOLBERT[6].

Qualitatively, regardless of the conditions of acid and heat treatment of the sedoheptulosan solution (Table I), all samples showed a similar three-component chromatographic pattern (I, II, III). Component (IV) appeared on chromatograms in large amount from the samples treated 125 h at 80° and 50 h at 100°. Much smaller amounts of this component appeared from the samples treated 4 h at 80°, and 1 h at 100°, and only a trace amount in the sample treated 40 h at 60°. Under the conditions of the experiment, no detectable amounts of (IV) were observed with the samples treated 140 h at 40° or at room temperature (25°), 240 h.

Of the irrigants listed in Table II and for the times of irrigation indicated, only the *n*-butanol–propionic acid–water mixture retained a solvent front; the other mixtures had dripped off the edge of the paper. Chromatograms were irrigated for shorter periods with the faster irrigants, and thereby retained their solvent fronts, confirming the fact that component (IV) follows closely the solvent front. Except as noted in Table II, no comparable migration value could be given for component (IV) and simultaneously obtain good separation of the other components.

The values for the migration distances of the sugars shown in Table II may vary from experiment to experiment but, if chromatographic conditions are rigidly controlled, the relative differences as shown should prevail. The ethyl acetate mixture should be freshly prepared for each experiment, because of the tendency for this mixture to change its irrigating properties. The procedure required for preparing the *n*-butanol–propionic acid–water mixture[8] does not recommend it for common usage.

If mannoheptulose is included, it will also appear on the orcinol-treated chromatogram within one minute of heating at 105°. Dependent upon the experimental conditions (type of chromatographic paper and composition of irrigant mixture) the migration rates of the mannoheptulose and sedoheptulosan (II) spots may be the same. This factor could be problematical if an unknown solution were to contain both components. However, in this study, at least two of the chromatographic papers—Eaton and Dikeman* Nos. 4 and 5—showed considerable promise in producing distinct differences in the migration rates of mannoheptulose and sedoheptulosan (II) when the mixture of ethyl acetate, pyridine, and water (8:2:1) was used for 16 h at 25° (Table II). The data also showed that the two spots considered to be 2,7-anhydro-β-D-altro-heptulofuranose (III) and 5-(1,2-dihydroxyethyl)-2-furaldehyde (IV) moved considerably faster than the other sugars mentioned regardless of the type of paper or irrigant used.

ACKNOWLEDGEMENT

We are indebted to Dr. NELSON K. RICHTMYER of the National Institutes of Health, Bethesda, Maryland, for the samples of sedoheptulosan monohydrate and the *Sedum* sirup.

SUMMARY

A maximum of four components were found by paper chromatography in an acid-treated solution of sedoheptulosan. One of the components, 5-(1,2-dihydroxyethyl)-2-furaldehyde, follows closely the solvent front on the chromatogram.

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* Mention of specific products does not imply recommendation by the Department of Agriculture over others of a similar nature not mentioned.

Sedoheptulose appeared much more quickly than sedoheptulosan (2,7-anhydro-β-D-altro-heptulopyranose) and its second anhydride (2,7-anhydro-β-D-altro-heptulofuranose) when the paper chromatograms treated with orcinol were heated.

Mannoheptulose, when treated with the orcinol reagent, will also appear at approximately the same time as sedoheptulose on the chromatogram.

Eaton and Dikeman chromatographic papers, Nos. 4 and 5, were most effective in the separation of mannoheptulose, sedoheptulose, and sedoheptulosan, using the solvent mixture ethyl acetate–pyridine–water (8:2:1).

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SUBSTANCES POLYPHÉNOLIQUES DES PLANTES

II. SÉPARATION DES ACIDES PHÉNOLIQUES DU CAFÉ VERT ET DU CAFÉ RÔTI*

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I. INTRODUCTION

Les recherches chimiques effectuées depuis le début du 19e siècle sur les fèves de café ont mis en évidence la présence d'un certain nombre de substances polyphénoliques.

Le premier, PAYEN[1] réussit à isoler, à partir du café vert, le sel complexe de potassium et de caféine d'un acide phénolique, qu'il baptisa "acide chlorogénique". Un demi siècle plus tard, GRIEBEL[2] obtint à partir de ce complexe l'acide lui-même, sous forme cristallisée. GORTER[3], FREUDENBERG[4], puis FISCHER ET DANGSCHAT[5] continuèrent l'étude de l'acide chlorogénique, se préoccupant plus particulièrement de la structure de ce composé. Ils prouvèrent que l'acide chlorogénique est un depside de l'acide caféique avec l'acide quinique, l'acide caféoyl-3-quinique.

Quelques années plus tard, BARNES *et al.*[6] découvrirent la présence dans les fèves de café vert d'un autre depside des acides caféique et quinique, qu'ils appelèrent "acide isochlorogénique". Ils proposèrent pour la constitution de cette nouvelle substance une structure d'acide caféoyl-5-quinique. Cette hypothèse a été combattue par URITANI ET MIYANO[7], qui lui opposèrent une structure d'acide caféoyl-4-quinique.

Un troisième depside des acides caféique et quinique a été isolé de la purée de pêches par CORSE[8], qui le nomma "acide néochlorogénique". Cet acide a été ensuite décelé dans le café vert par SONDHEIMER[9], et les travaux de LENTNER ET DEATHERAGE[10] ont confirmé cette découverte. Nos recherches nous ont également permis de vérifier la présence de l'acide néochlorogénique dans le café vert et le café rôti. Aucune formule de constitution de l'acide néochlorogénique n'a été proposée jusqu'à présent.

Le présent article décrit une méthode d'extraction des acides phénoliques du café (vert ou rôti), une étude par chromatographie sur papier des extraits ainsi obtenus et des essais de séparation des constituants de ces extraits par chromatographie de partage sur colonne.

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* Première communication dans cette série réf.[19].
** Directeur: Dr. R. H. EGLI.

II. PARTIE GÉNÉRALE

A. Extraction des acides phénoliques

1. *Méthodes existantes*

Les auteurs, qui ont étudié l'acide chlorogénique du café vert, ont employé des méthodes d'extraction, très semblables quant à leur déroulement, que nous pouvons résumer de la façon suivante:

Extraction des fèves de café vert à l'eau ou au moyen d'un solvant aqueux.

Libération des extraits aqueux de la caféine et des matières grasses.

Précipitation de l'acide chlorogénique sous forme de son sel de plomb ou de son complexe de caféine et de potassium, séparés de l'extrait aqueux par filtration.

Régénération de l'acide chlorogénique à partir de son sel de plomb ou de son complexe, purification et recristallisation.

Après avoir utilisé deux des méthodes d'extraction les plus récentes, celle de FIEDLER[11] et celle de KREMERS (rapportée dans un article de MOORES et coll.[12]), nous avons établi un procédé plus pratique, qui n'est qu'une adaptation des deux méthodes mentionnées.

2. *Extraction des acides phénoliques du café*

Extraction du café finement moulu (vert ou rôti) à l'alcool isopropylique aqueux.

Evaporation sous vide du solvant organique.

Elimination des graisses coagulées par filtration de l'extrait aqueux sur un lit de cellite.

Addition d'une solution saturée d'acétate de plomb au filtrat. Le pH de la suspension est porté á 8.0 au moyen d'une solution de soude caustique.

Filtration du précipité obtenu, lavage sur le filtre et séchage.

Régénération des acides phénoliques à partir des sels de plomb au moyen d'une solution d'acide sulfurique.

Lyophilisation de la solution aqueuse des acides régénérés.

Cette méthode d'extraction a donné d'excellents résultats. A partir d'un kilo de café de Colombie, nous avons obtenu 62.5 g d'acides phénoliques dans le cas du café vert et 35.5 g dans le cas du café rôti.

Pour étudier de façon plus complète les substances phénoliques du café vert, nous avons légèrement modifié notre méthode d'extraction. La précipitation des sels de plomb des acides phénoliques a été effectuée en deux stades, le premier à pH 5.0, le second à pH 8.0. Chaque précipité a été traité de façon habituelle et a donné, après régénération, une fraction distincte d'acides phénoliques lyophilisés.

Cette modification de la méthode d'extraction constitue une préséparation partielle des acides phénoliques du café vert; certains d'entre eux ne sont présents que dans une des fractions envisagées (voir Figs. 2 et 3).

B. Chromatographie sur papier

Les mélanges des acides phénoliques extraits du café (vert ou rôti) ont été examinés par chromatographie sur papier bidimensionnelle.

Les conditions de cette méthode, décrites par ROBERTS[13,15], sont reproduites dans la PARTIE EXPÉRIMENTALE, ainsi que les méthodes utilisées pour l'identification des constituants du mélange phénolique.

Huit acides phénoliques ont été identifiés dans les mélanges extraits du café vert et du café rôti. Le Tableau I contient les noms de ces différents acides, ainsi que quelques-unes de leurs propriétés chromatographiques.

La Fig. 1 reproduit un chromatogramme des acides phénoliques extraits du café rôti. Les Fig. 2 et 3 représentent des chromatogrammes des deux fractions des acides phénoliques extraits du café vert.

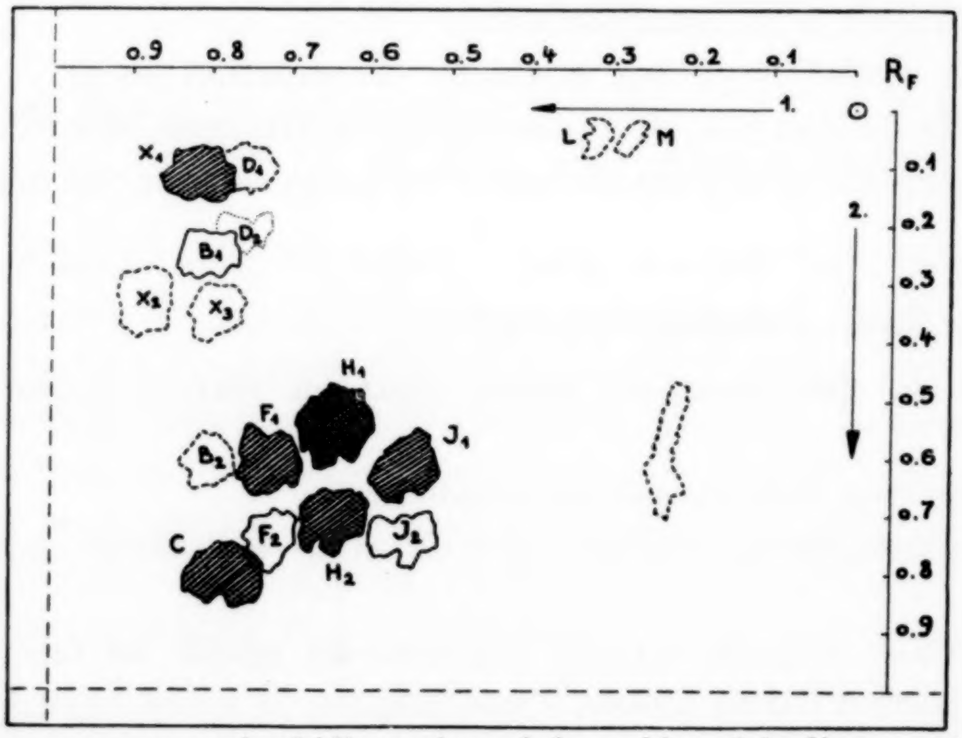

Fig. 1. Chromatogramme sur papier bidimensionnel des acides phénoliques extraits du café rôti.

L'examen par chromatographie sur papier des mélanges d'acides phénoliques extraits du café vert et du café rôti nous conduit aux constatations suivantes:

(a) Les acides chlorogéniques, c'est à dire les depsides de l'acide caféique avec l'acide quinique, constituent la majeure partie de la fraction phénolique du café.

(b) A côté des acides chlorogéniques, cette fraction contient, en moindre quantité, trois substances que nous supposons être des acides féruloylquiniques, c'est à dire des depsides de l'acide férulique avec l'acide quinique. Ces substances se comportent chimiquement comme les acides chlorogéniques et procurent, par hydrolyse, l'acide férulique et l'acide quinique*.

(c) Dans les extraits du café "robusta" provenant d'Angola, des quantités assez importantes d'acide *p*-coumarique ont été décelées. Cette substance ne se retrouve dans aucun des cafés "arabica" étudiés au cours des présents travaux*.

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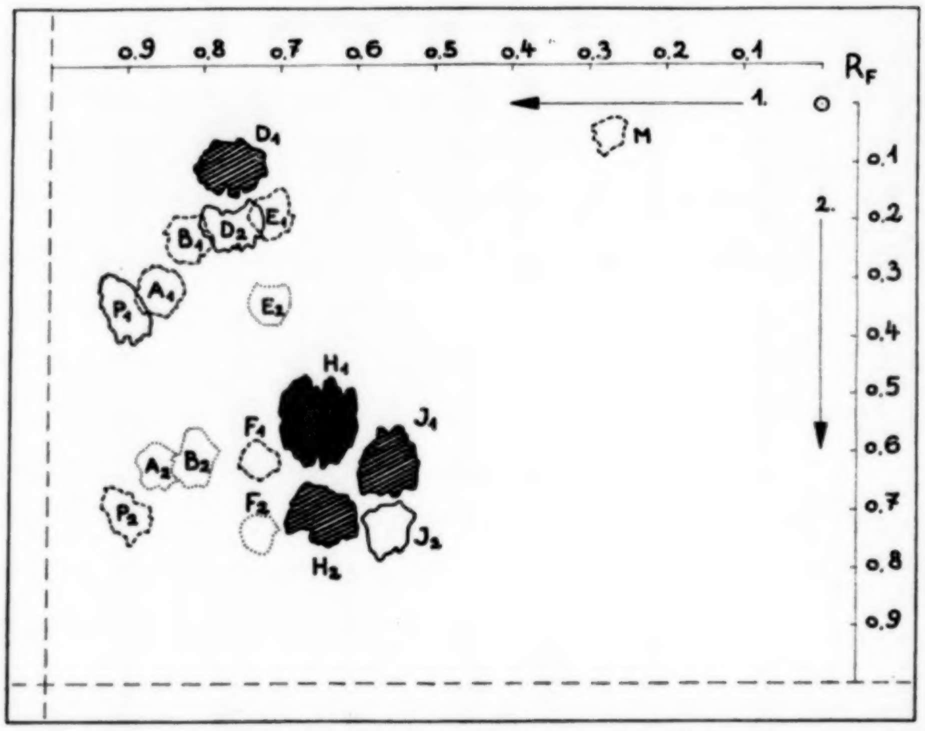
* Pendant la rédaction de ce travail un nouvel article de LENTNER ET DEATHERAGE a paru[20], mentionnant la présence dans le café, vert ou rôti, de deux acides féruloylquiniques et d'un acide *p*-coumarylquinique.

TABLEAU I

LES ACIDES PHÉNOLIQUES ET LEURS PROPRIÉTÉS CHROMATOGRAPHIQUES

Code sur chromatogrammes	*Substance*	*R_F avec n-butanol–acide acétique–eau (4:1:2.2)*		*R_F avec acide acétique 2 %*		*Fluorescence U.V.*	*Coloration avec benzidine bisdiazotée*	*Apparition de la coloration*
		Littérature	*Nos valeurs*	*Littérature*	*Nos valeurs*			
A_1 et A_2	Acide férulique	0.84[18]	0.84–0.86	0.38[16] 0.65[16]	0.33–0.34 0.64–0.65	Bleue	Rouge-brun	Lente
B_1 et B_2	Acide caféique	0.78[18]	0.78–0.80	0.30[16] 0.63[16]	0.25–0.26 0.61–0.63	Vert-clair	Rouge-brique	Normale
C	Caféine	—	0.76–0.77	—	0.80–0.81	Violette	—	—
D_1 et D_2	Acide isochlorogénique	0.76[15]	0.74–0.75	0.11[15] 0.21[15]	0.11–0.13 0.22–0.24	Vert-clair	Jaune-brun	Instantanée
E_1 et E_2	Acide féruloylquinique I	—	0.70–0.72	— —	0.19–0.20 0.35–0.36	Bleu-sombre	Rouge-brun	Lente
F_1 et F_2	Acide féruloylquinique II	—	0.72–0.73	— —	0.61–0.62 0.74–0.75	Bleue	Rouge-brun	Lente
G_1 et G_2	Acide féruloylquinique III	—	0.67–0.69	— —	0.64–0.65 0.78–0.79	Bleu-sombre	Rouge-brun	Lente
H_1 et H_2	Acide chlorogénique	0.64[15]	0.62–0.64	0.56[15] 0.72[15]	0.55–0.57 0.72–0.74	Vert-clair	Jaune-brun	Instantanée
J_1 et J_2	Acide néochlorogénique	0.55[15]	0.53–0.55	0.63[15] 0.74[15]	0.62–0.64 0.74–0.75	Vert-clair	Jaune-brun	Instantanée


0.9
0.8
0.7
0.6
0.5
0.4
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0.1
RF
1.
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D1
B1
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A1
P1
E2
H1
F1
J1
A2
B2
F2
P2
H2
J2
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

Fig. 2. Chromatogramme sur papier bidimensionnel des acides phénoliques extraits du café vert et dont les sels de plomb ont été précipités à pH 5.0. *Note*: Les taches P_1 et P_2 correspondent à l'acide *p*-coumarique présent dans le café "robusta" d'Angola.

(d) La présence des acides caféique et férulique dans plusieurs de nos chromatogrammes de café vert n'est due qu'à l'hydrolyse de leurs depsides pendant le traitement. L'acide caféique[17] ou l'acide férulique libres ne se trouvent pas dans le café vert ou le café rôti.

(e) Chaque acide phénolique se sépare en deux taches sous l'influence du solvant de seconde dimension (acide acétique 2%). Ce phénomène avait déjà été mis en évidence par WILLIAMS[16] et par ROBERTS[15], qui pensent qu'il s'agit d'une isomérie "*cis–trans*". Nous poursuivons l'étude de ce dédoublement chromatographique.

(f) A part les acides chlorogéniques et les acides féruloylquiniques, la fraction phénolique du café recèle d'autres substances, le plus souvent à l'état de traces:

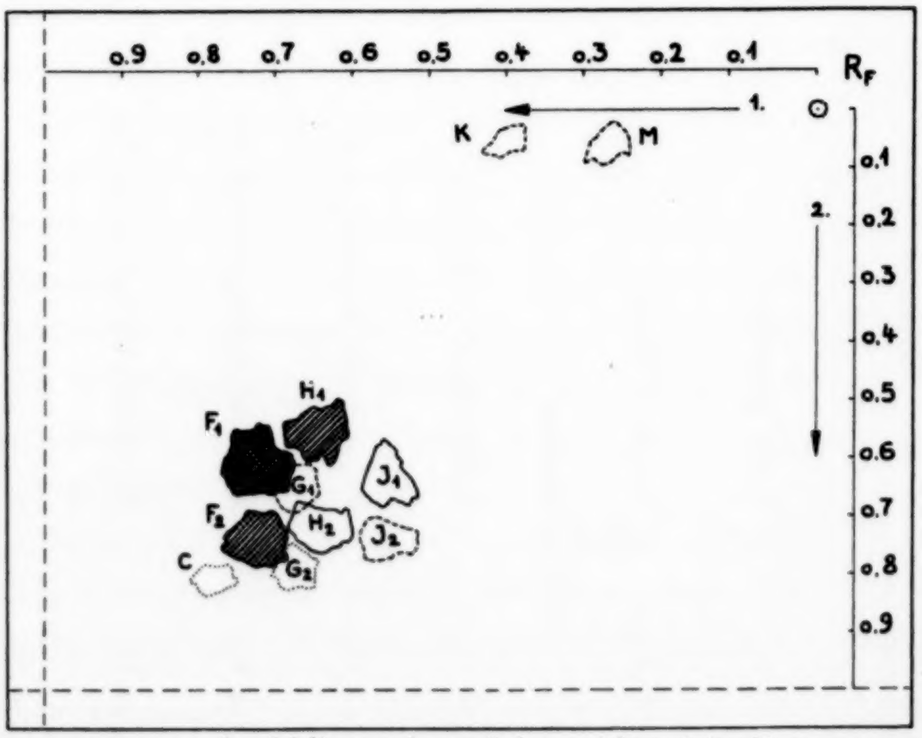
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
RF
1.
2.
K
M
H1
F1
G1
J1
F2
H2
J2
C
G2
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9

Fig. 3. Chromatogramme sur papier bidimensionnel des acides phénoliques extraits du café vert et dont les sels de plomb ont été précipités à pH 8.0.

(1) La caféine, liée par complexe au chlorogénate de potassium[3,17], complexe très probablement coprécipité avec les sels de plomb.

(2) Des substances inconnues ne présentant pas les caractères phénoliques, mais produisant une fluorescence jaune à l'irradiation U.V. des chromatogrammes. Dans nos chromatogrammes, ces substances sont désignées par la lettre majuscule X, flanquée d'un indice de numérotation. Elles n'ont été remarquées que dans les chromatogrammes de la fraction phénolique du café rôti.

(3) Des substances phénoliques, faiblement colorées, qui se déplacent peu sous l'influence des solvants de première et de seconde dimensions. Elles sont présentes en beaucoup plus grande quantité dans les extraits de café rôti que dans ceux de café vert. Il semble s'agir de produits de condensation, de polymérisation et/ou d'oxydation des acides phénoliques, formés en majeure partie au cours de la torréfaction. Dans nos chromatogrammes, ces substances sont désignées par les lettres majuscules K, L, M et N.

C. Chromatographie de partage sur colonne

1. *Méthode de chromatographie de partage des acides phénoliques*

Des essais de séparation des constituants des mélanges d'acides phénoliques ont été effectués par chromatographie de partage sur colonne de cellulose, en suivant les prescriptions suivantes[19]:

Phase stationnaire: eau distillée fixée sur poudre de cellulose.

Phase mobile pour les acides phénoliques du café vert, dans l'ordre: propionate d'éthyle – acétate d'éthyle – lavage de la colonne au butanol.

Phase mobile pour les acides phénoliques du café rôti, dans l'ordre: propionate d'éthyle contenant 10 % de benzine – propionate d'éthyle – acétate d'éthyle – butanol – lavage de la colonne au méthanol.

Les solvants de la phase mobile sont recueillis par fractions de volume égal, dont l'absorption lumineuse est mesurée à 322 mμ, cette longueur d'onde représentant un maximum d'absorption pour presque tous les acides phénoliques présents dans le mélange. On reproduit graphiquement l'absorption U.V. des fraction successives en fonction de l'écoulement et collationne en fractions "principales".

Le contenu de chaque fraction principale est étudié par chromatographie sur papier, ce qui permet de juger de l'efficacité de la partition chromatographique pour la séparation.

2. *Acides phénoliques du café vert*

Les acides phénoliques, contenus dans le café vert et isolés selon la méthode d'extraction décrite précédemment, ont été soumis à la chromatographie de partage sur colonne de cellulose. L'absorption des différentes fractions à 322 mμ fournit un bilan d'élution entre 95 et 100 %. Le reste a été élué de la colonne par du butanol normal à demi saturé d'eau.

La Fig. 4 représente la courbe de l'absorption des différentes fractions à 322 mμ. La Fig. 5 représente les chromatogrammes sur papier monodimensionnels des diverses

TABLEAU II

SÉPARATION DES ACIDES PHÉNOLIQUES DU CAFÉ VERT

Fraction	*Principal constituant*	*Constituant secondaire*	*% approx.*
I	Acide isochlorogénique	Acide caféique	0.9
II	Acide isochlorogénique	Acide féruloylquinique I	5.5
III	Acide féruloylquinique I	Acide isochlorogénique	13.2
IV	Acide féruloylquinique II	Traces de caféine	4.7
V	Acide chlorogénique	Acide féruloylquinique II	2.2
VI	Acide chlorogénique	Traces de caféine	52.6
VII	Acide chlorogénique	Traces ac. féruloylquinique III	10.3
VIII	Acide chlorogénique	Acide féruloylquinique III	1.7
IX	Acide néochlorogénique	Acide chlorogénique	2.3
X	Acide néochlorogénique	—	6.8
Reste	Substances K, L, M et N	Acide néochlorogénique	—

fractions principales, en utilisant comme solvant le mélange butanol–acide acétique–eau (4:1:2.2). Les acides phénoliques révélés chromatographiquement dans chaque fraction sont énumérés dans le Tableau II.

Les résultats de cette chromatographie de partage amènent aux conclusions suivantes:

Une séparation partielle des acides phénoliques du café vert est effectuée par la chromatographie de partage sur colonne de cellulose. Cette chromatographie fournit des indications intéressantes sur les proportions relatives de chaque constituant du mélange phénolique.

Certains acides phénoliques sont très difficiles à séparer les uns des autres. C'est le cas pour l'acide caféique (s'il est présent dans le mélange) d'avec l'acide isochlorogénique, et de l'acide isochlorogénique d'avec l'acide féruloylquinique I. L'acide féruloylquinique III n'a pas pu être séparé de l'acide chlorogénique proprement dit.

V.4
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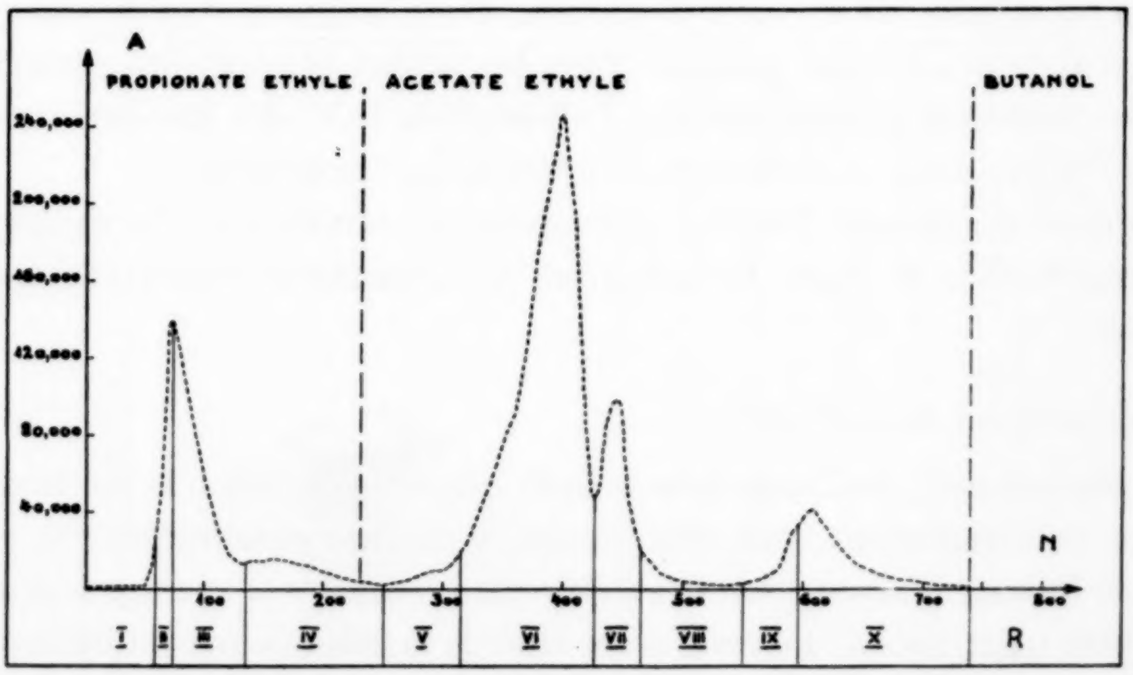

Fig. 4. Schéma de la séparation des acides phénoliques extraits du café vert. En abscisse, le numéro d'ordre des fractions d'élution. En ordonnée, les valeurs de l'absorption lumineuse de ces fractions à 322 mμ. *Note*: La répartition des fractions d'élution en fractions principales est indiquée au moyen des chiffres romains.

Comme on peut le constater sur la Fig. 4, l'acide chlorogénique se partage en deux "peaks" distincts. Ce "double peaking", qui n'est pas une conséquence de l'isomérisation *cis–trans* de l'acide, n'a pas pu être expliqué jusqu'à présent de façon satisfaisante.

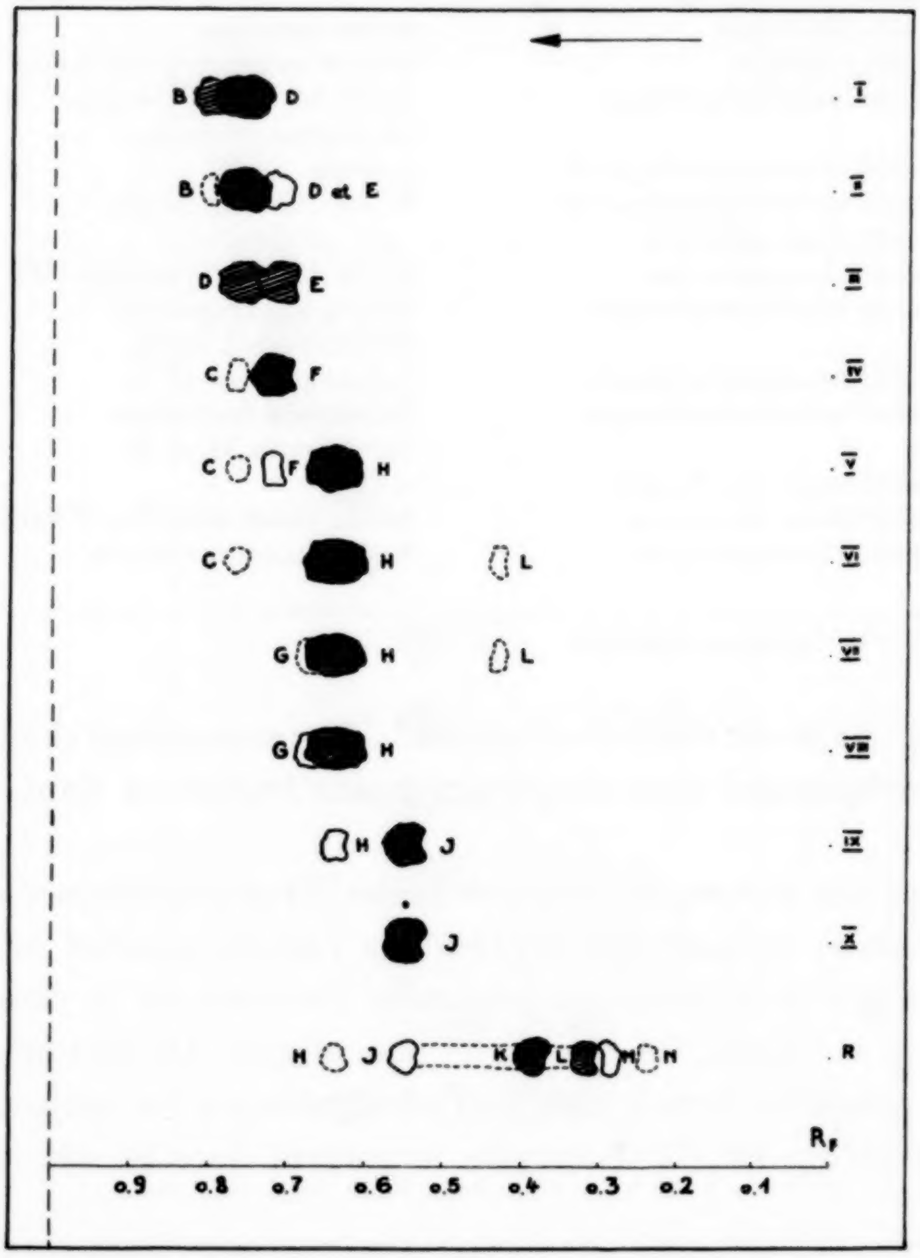

Fig. 5. Chromatogramme sur papier monodimensionnel des acides phénoliques du café vert, contenus dans les diverses fractions principales de la séparation.

3. *Acides phénoliques du café rôti*

Les acides phénoliques du café rôti ont également été soumis à la chromatographie de partage sur colonne de cellulose. L'absorption U.V. des fractions à 322 mμ fournit un bilan d'élution entre 95 et 100 %. Le reste a été élué au méthanol contenant 10 % d'eau.

La Fig. 6 représente la courbe de l'absorption des différentes fractions à 322 mμ. La Fig. 7 représente les chromatogrammes sur papier monodimensionnels des différentes fractions principales, le solvant utilisé étant le mélange butanol–acide acétique–eau (4:1:2.2). Les acides phénoliques révélés chromatographiquement dans chaque fraction sont énumérés dans le Tableau III.

Les conclusions suggérées par les résultats de cette séparation des acides phénoliques du café rôti sont les suivantes:

Une séparation partielle des acides phénoliques du café rôti à l'aide de la chro-

TABLEAU III

SÉPARATION DES ACIDES PHÉNOLIQUES DU CAFÉ RÔTI

Fraction	*Principal constituant*	*Constituant secondaire*	*% approx.*
I	Acide férulique	Acide caféique	0.1
II	Acide caféique	Traces substance inconnue	3.9
III	Acide isochlorogénique	Acide féruloylquinique I et substance inconnue	1.3
IV	Acide féruloylquinique II	Caféine	6.0
V	Acide féruloylquinique II	Acide chlorogénique	3.5
VI	Acide chlorogénique	—	19.0
VII	Acide chlorogénique	Acide féruloylquinique III	28.8
VIII	Acide néochlorogénique	Substance inconnue Substances L et M	10.2
IX	Acide néochlorogénique	Substances M et N	13.8
X	Acide néochlorogénique	Substance inconnue Substances M et N	2.1
XI	Substances inconnues	—	3.5
XII-XIII	Substances inconnues	Acide chlorogénique (Fraction XII)	7.8
Reste	Acide chlorogénique*	Substances inconnues	—

* Probablement sous forme complexée.

matographie de partage sur colonne est possible. Le pourcentage des acides isochlorogénique et féruloylquinique I est beaucoup moins important dans le café rôti que dans le café vert.

A côté des acides chlorogéniques, des acides féruloylquiniques et de la caféine, la fraction phénolique du café rôti contient un certain nombre de substances non déterminées. Il s'agit de substances présumées provenir de la condensation, de la polymérisation ou de l'oxydation des acides phénoliques. On distingue d'une part les substances déjà présentes dans le café vert (désignées par les majuscules K, L, M et N), dont le pourcentage est d'ailleurs plus important dans le café rôti, d'autre part

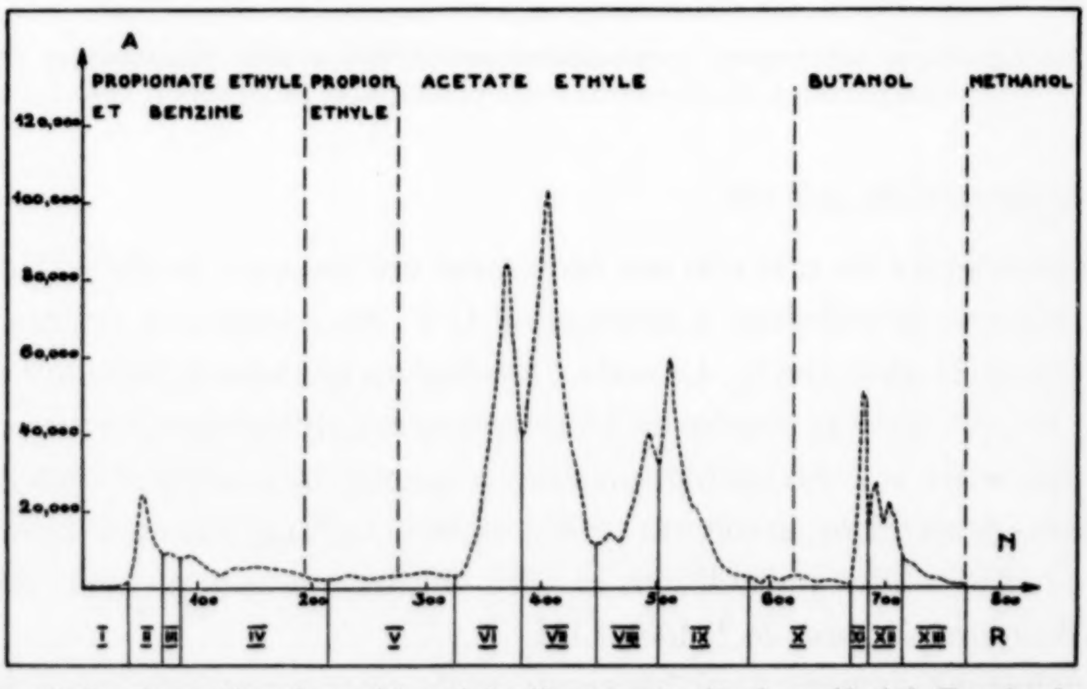

Fig. 6. Schéma de la séparation des acides phénoliques extraits du café rôti. En abscisse, le numéro d'ordre des fractions d'élution. En ordonnée les valeurs de l'absorption lumineuse de ces fractions à 322 mμ. *Note*: La répartition des fractions d'élution en fractions principales est indiquée au moyen des chiffres romains.

les substances non présentes dans le café vert (désignées par la majuscule X, flanquée d'un indice de numérotation).

De même que dans le café vert, certains acides phénoliques sont difficiles à séparer les uns des autres. L'acide féruloylquinique III, présent en très petites quantités, n'a pas pu être séparé de l'acide chlorogénique.

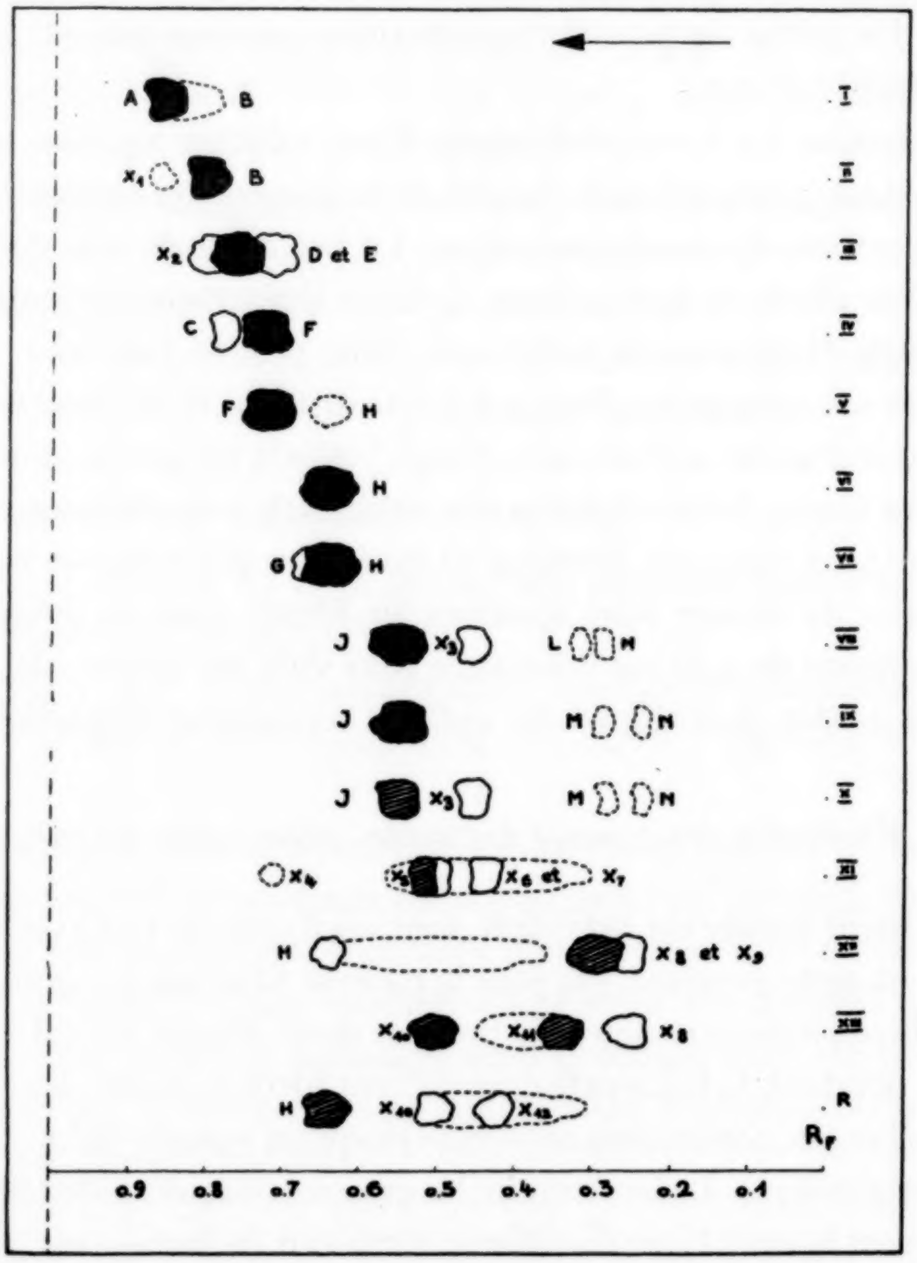

Fig. 7. Chromatogramme sur papier monodimensionnel des acides phénoliques du café rôti, contenus dans les diverses fractions principales de la séparation.

Les acides chlorogénique et néochlorogénique présentent le phénomène du "double peaking", remarqué lors de la séparation des acides phénoliques du café vert.

III. PARTIE EXPÉRIMENTALE

A. Extraction des acides phénoliques du café

1 kg de café finement moulu (vert ou rôti) est introduit dans un flacon de 10 l, et 6 l d'isopropanol 70 % sont ajoutés. Le flacon est placé dans une secoueuse pendant 3 h. Le café est alors filtré sur un grand Büchner, puis replacé dans le flacon avec une nouvelle portion de 6 l d'isopropanol 70 % et agité pendant ½ h. Le café épuisé est

filtré sur Büchner, puis écarté. Les extraits isopropanoliques sont mélangés, concentrés sous vide jusqu'au volume de 2 l (élimination complète du solvant organique) puis laissés reposer pendant une nuit au frigorifique. Le lendemain, les graisses contenues dans l'extrait et coagulées pendant la nuit, sont filtrées sur un lit de cellite, préparé sur un Büchner. La cellite est lavée à l'eau distillée (environ 500 ml) et l'eau de lavage est ajoutée au filtrat.

Le filtrat (environ 2.5 l) est additionné d'une solution aqueuse saturée d'acétate de plomb (200 g dans 400 ml d'eau). Le pH de la suspension est porté à 8.0 au moyen d'une solution normale de soude caustique. Le précipité de sels de plomb est filtré sur un grand filtre plissé et lavé à l'eau distillée légèrement carbonatée (pH 8.0), le tout sous agitation. Il est ensuite séché sous vide, pesé et pulvérisé.

Cette poudre est suspendue dans 1 l d'eau distillée et la suspension additionnée de petites portions d'acide sulfurique normal, jusqu'à ce que la couleur du précipité passe du jaune au blanc. Cette régénération se déroule sous forte agitation. Le sulfate de plomb précipité est filtré sur Büchner et lavé avec une solution très diluée d'acide acétique. Les eaux de lavage sont ajoutées au filtrat, puis ce mélange (dont le pH doit être aux environs de 4.5) est concentré sous vide au volume de 300 ml. La solution aqueuse des acides phénoliques du café est finalement lyophilisée.

B. Extraction fractionnée des acides phénoliques du café vert

1 kg de café finement moulu est introduit dans un flacon de 10 l avec 6 l d'isopropanol 70 %. Le flacon est agité pendant 3 h, puis le café est filtré sur un grand Büchner. Il est replacé dans le flacon avec une nouvelle portion de 6 l d'isopropanol 70 % et le tout est agité à nouveau pendant ½ h. Le café épuisé étant filtré et écarté, les extraits isopropanoliques sont mélangés, concentrés sous vide jusqu'au volume de 2 l, puis laissés reposer une nuit au frigorifique. Le lendemain, les graisses coagulées sont filtrées sur un lit de cellite. La cellite est lavée à l'eau distillée et cette eau de lavage est ajoutée au filtrat.

Ce filtrat est alors additionné d'une solution saturée d'acétate de plomb. Le pH de la suspension ainsi obtenue est porté à 5.0 au moyen d'une solution diluée de soude caustique. Le précipité est filtré sur un grand filtre plissé, sous agitation, et lavé sur le filtre avec 2 l d'eau distillée légèrement acidifiée à l'acide acétique. Le filtrat et les eaux de lavage sont mélangés, concentrés sous vide au volume de 1.5 l. Le pH de cette solution est alors ramené à 8.0 au moyen d'une solution normale de soude. Le nouveau précipité est filtré et lavé comme le précédent.

Les deux précipités de sels de plomb sont séparément séchés sous vide, pesés, pulvérisés finement, puis suspendus dans 1 l d'eau distillée. Chacune de ces suspensions est alors additionnée, sous forte agitation, de petites portions d'acide sulfurique normal, jusqu'à changement de couleur du précipité. Le sulfate de plomb est filtré sur Büchner et lavé avec une solution diluée d'acide acétique. Les eaux de lavage sont ajoutées au filtrat, puis ces mélanges (dont le pH est aux environs de 4.5) sont concentrés sous vide au volume de 300 ml. Ces deux solutions aqueuses des acides phénoliques du café vert sont lyophilisées.

C. Chromatographie sur papier

1. *Chromatographie des acides phénoliques*

Mode de chromatographie:	bidimensionnelle descendante
Papier utilisé:	Whatman No. 1
Poids d'extrait sec déposé:	150 à 300 γ selon le nombre de composants
Solvant de première dimension:	*n*-butanol–acide acétique–eau (4:1:2.2)
Solvant de seconde dimension:	acide acétique 2 %
Révélation des taches:	(a) fluorescence U.V. (b) vaporisation d'une solution aqueuse de benzidine bisdiazotée.

2. *Identification des substances responsables des taches*

(a) Comparaison avec les R_F de la littérature, en ayant soin d'utiliser un solvant de première dimension fraîchement préparé.

(b) Comparaison par chromatographie monodimensionnelle, en utilisant notre solvant de première dimension, avec des acides phénoliques du commerce recristallisés.

(c) Chromatographie de superposition, en surchargeant les taches de départ de nos chromatogrammes bidimensionnels au moyen de 50 γ d'un acide phénolique connu et purifié, qui peut soit surcharger, soit créer une ou plusieurs taches.

(d) Elution des taches à l'eau distillée, après avoir été décelées par fluorescence U.V. et découpées. Les solutions aqueuses ainsi obtenues sont étudiées par spectrophotométrie U.V. et par hydrolyse, les produits de ces hydrolyses étant identifiés par chromatographie sur papier (Fig. 8).

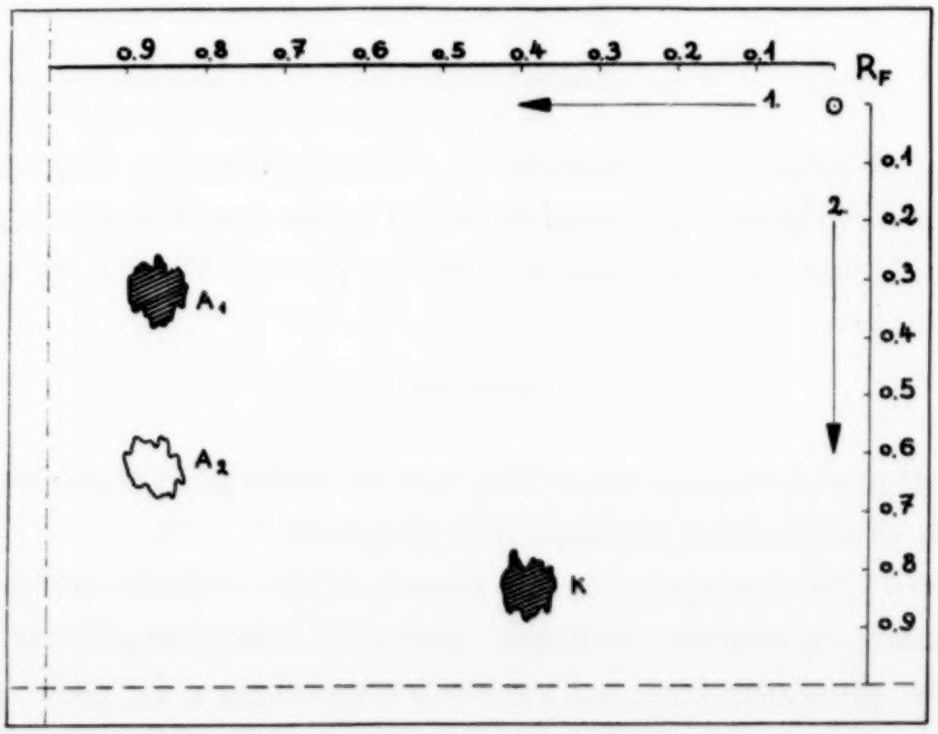

Fig. 8. Chromatogramme sur papier bidimensionnel des substances obtenues par hydrolyse de l'acide féruloylquinique II. A_1 et A_2 = acide férulique. K = acide quinique.

D. Chromatographie de partage des acides phénoliques

(*a*) *Préparation de la colonne et dépôt du mélange à séparer*

200 g de poudre de cellulose Whatman sont suspendus dans du propionate d'éthyle saturé d'eau et contenant 10 % de benzine. 100 g d'eau distillée sont ajoutés par petites portions, en prenant soin d'agiter très vigoureusement la suspension. Cette suspension est ensuite utilisée pour remplir une colonne de 60 × 4 cm.

2.5 g d'acides phénoliques lyophilisés sont mélangés intimement dans un mortier à 10 g de cellulose sèche, humidifiés puis déposés en haut de la colonne[19].

(*b*) *Développement de la colonne et analyse de l'éluat*

La colonne est alimentée avec un solvant organique, dans l'ordre:

Mélange propionate d'éthyle–benzine (9:1), saturé d'eau.
Propionate d'éthyle saturé d'eau.
Acétate d'éthyle saturé d'eau aux deux tiers.
n-Butanol à demi saturé d'eau.
Méthanol contenant 10 % d'eau.

Ce solvant provient d'un mélangeur continu, qui fournit un gradient de concentration progressif lors des changements de solvant[19]. Après passage dans la colonne, le solvant d'élution est recueilli dans les éprouvettes d'un collecteur de fractions, à raison de 300 goutes par éprouvette. L'absorption à 322 mμ de ces fractions est mesurée et reportée sur un graphique, permettant de collationner l'éluat en fractions principales. Le volume des fractions principales est également mesuré, en vue du calcul du bilan d'élution (basé sur l'extinction U.V. des fractions). Chaque fraction principale est concentrée sous vide à un petit volume, additionnée d'eau, puis libérée par évaporation sous vide du reste du solvant organique. Chacune de ces solutions aqueuses est examinée par chromatographie sur papier monodimensionnelle, puis lyophilisée.

REMERCIEMENTS

Nous tenons à remercier ici le docteur J. CORSE (Western Regional Laboratory, Albany, Calif., U.S.A.) pour son aimable don d'acide néochlorogénique.

Nos remerciements vont également à Melle. H. VON BÜREN, pour son assistance intelligente et efficace.

RÉSUMÉ

Les acides phénoliques des cafés vert et rôti ont été isolés par extraction à l'isopropanol aqueux, puis par précipitation de leurs sels de plomb.

Une étude par chromatographie sur papier de ces extraits phénoliques a permis d'identifier les acides suivants: férulique, caféique, isochlorogénique, chlorogénique, néochlorogénique, ainsi que trois acides féruloylquiniques. Le café "robusta" provenant d'Angola contient en plus une certaine quantité d'acide *p*-coumarique.

Quant aux séparations des acides phénoliques par chromatographie de partage

sur colonne de cellulose, si elles ne furent pas aussi complètes que nous le désirions, elles se sont montrées fort efficaces et ont fourni d'utiles indications sur les proportions respectives desdits acides dans les cafés vert et rôti.

SUMMARY

The phenolic acids of green and roasted coffee were extracted with aqueous isopropanol and isolated by precipitating their lead salts.

The following acids could be identified by paper chromatography: ferulic, caffeic, isochlorogenic, chlorogenic, neochlorogenic and three ferulylquinic acids. "Robusta" coffee contains, in addition, *p*-coumaric acid.

The main components of the phenolic fractions of green and roasted coffee were isolated by partition chromatography on cellulose powder columns. A good estimate of the quantitative composition of the extracts was obtained.

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IMPROVED METHODS FOR THE PAPER CHROMATOGRAPHY OF ORGANIC ACIDS

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In the course of research into the chemical constitution of coal, simple, reliable routine methods were required for the chromatographic examination of the complex mixtures of aliphatic and aromatic acids produced by oxidative degradation of coals. The present work was carried out to find acidic, basic, and buffered solvent mixtures suitable for the separation of the following acids (referred to later as "reference acids"), which were chosen as representative of the types to be expected to result from oxidation of coals: oxalic, malonic, glutaric, L-tartaric, citric, phthalic, 1-naphthoic, and gallic acids. Many solvent mixtures used previously by other workers were tried, but without success; in particular, oxalic acid, often produced by oxidative degradation of organic compounds, was found in almost every case to form a streak extending from the starting line.

Chromatography was carried out at room temperature on Whatman No. 1 paper by an ascending technique similar to that of WILLIAMS AND KIRBY[1]. In initial studies with solvent mixtures containing a considerable proportion of a volatile organic acid (the "swamping acid"), the papers were sprayed with an ethanolic solution of bromophenol blue adjusted to pH 6–7[2], when a parallel sided yellow acidic band was revealed which extended from the starting-edge of the paper and over its entire width. The band tended to obscure acid spots at lower R_F values, and many attempts were made, without success, to prevent its formation by pre-washing the paper in various ways. The formation of acid bands could be explained by postulating the existence in the paper of salts of which only the cations moved in the acid solvent, leaving a band of anions extending from the edge of the paper to the R_F value of the cations. In support of this explanation, the addition of sodium ions to the solvent completely removed the bands; the concentration of sodium ions had to be controlled so as to neutralise the anions without producing a corresponding blue basic band.

It was difficult to control the amount and distribution of an indicator applied to the chromatograms by spraying, and much better results were obtained by incorporating the indicator in the solvent; in this way an automatically-developed chromatogram was produced having a uniform background colour. This method had been tried earlier by REID AND LEDERER[3] and by WALKER[4]. To be effective it was necessary that the indicator used should have (a) a suitable pH range and (b) an R_F value of unity (or approaching unity) in the irrigating solvent. After examining many indicators, suitable ones were found for all the solvent mixtures used in the present work.

Three acid solvent mixtures were selected for routine work, containing ethyl formate or acetate, formic or acetic acid, and water. These mixtures are able to contain oxalic acid as a discrete spot. The selected basic solvent was a mixture of ethanol, ammonia, and water; LONG, QUAYLE AND STEDMAN[5] have used a solvent mixture containing the same substances but in different proportions. For the buffered solvents an ammonia-ammonium carbonate buffer (1.5 *N* with respect to each) was used. The compositions of the recommended solvents are shown below.

Solvent 1: Ethyl formate–98 % formic acid–water (12:5:3 v/v) containing bromophenol blue (0.015 % w/v) and sodium formate (0.05 % w/v).

Solvent 2: Ethyl formate–98 % formic acid–water (2:1:1 v/v) containing bromophenol blue (0.015 % w/v) and sodium formate (0.05 % w/v).

Solvent 3: Ethyl acetate–glacial acetic acid–water (2:1:1 v/v) containing bromophenol blue (0.015 % w/v) and sodium acetate (0.05 % w/v).

Solvent 4: Ethanol–water–0.880 ammonia (35:13:2 v/v) containing thymol blue (0.03 % w/v).

Solvent 5: Ethanol–buffer (7:3 v/v) containing chlorophenol red (0.03 % w/v).

Solvent 6: Ethanol–buffer (1:1 v/v) containing chlorophenol red (0.03 % w/v).

In the acid solvents 1 and 2 bromophenol blue moved with an R_F value of unity, while in solvent 3 the value was 0.95. Three or fours hours were required for the solvents to travel 20 cm. On leaving the chromatograms in the atmosphere overnight all the solvent evaporated, and the acids under examination were revealed as yellow spots on a uniform blue background. Methyl red, which had an R_F value of unity in all three acid solvents, could be used in place of bromophenol blue, but the visual contrast, between the red spots and the pink background, was less marked.

The basic solvent 4 required seven hours to travel 20 cm; the indicator, thymol blue, had an R_F value of 0.95. The acids under investigation were visible while the solvent was flowing, as yellow spots on a green background; these spots disappeared when the chromatogram was allowed to dry, but could be made to reappear, as faint red spots on a yellow background, by heating the chromatogram for 5 min in an oven at 150°.

The indicator incorporated in the buffered solvents, chlorophenol red, had an R_F value of 0.9 in solvent 5 and 0.95 in solvent 6. The chromatograms were allowed to dry in the air for about two hours, when the acids were revealed as yellow spots on a purple background. After about one hour further the spots faded, but could readily be regenerated by exposing the chromatograms to ammonia.

The general method outlined above is quite sensitive, since as little as 20 μg of an acid may be detected. Because of the uniform distribution of the indicator it was possible to employ the method in a roughly quantitative manner by comparing the spot produced by an unknown quantity of a particular acid with those produced by various known weights of the same material. Temperature had a small effect on R_F value, lower values being recorded at lower temperatures. Solvent flow was faster in the machine direction of the paper than in the perpendicular direction, but there was no detectable effect on R_F values. With some acids variation of the amount placed on the chromatogram was found to produce a small change in R_F value.

R_F values were obtained by measuring both to the front and to the rear edges of the spots; this method of measurement was preferred because it gave information on the length of a spot which could not be recorded by measuring to the centre. The R_F values of several organic acids in solvents 1–6 are shown in Table I, and examples of separations using solvent 1 are depicted in Fig. 1. It is proposed to publish at a later date the R_F values in solvents 1–6 of a wide variety of acids.

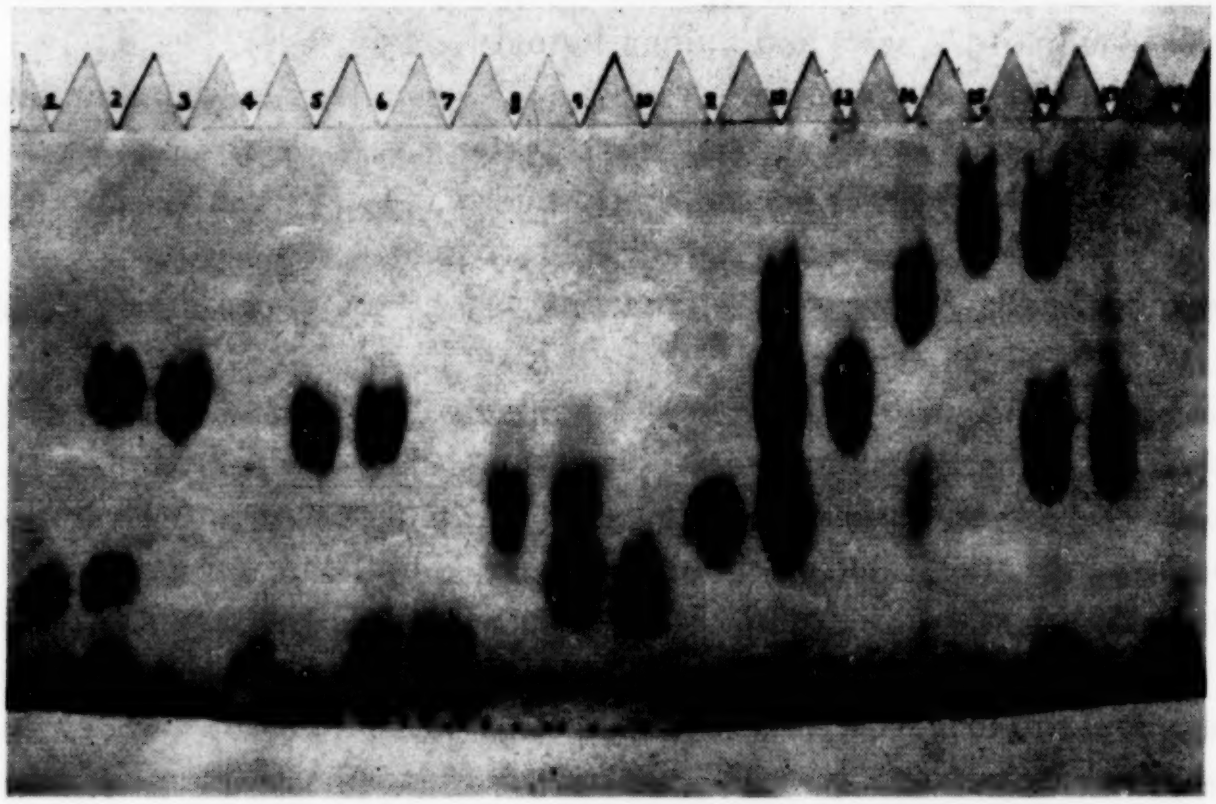

Fig. 1. Paper chromatography of non-volatile organic acids (solvent 1).

Position	*Acids*	*Position*	*Acids*
1	Malonic	12	Benzenepentacarboxylic,
2	L-Tartaric, malonic, phthalic		benzene-1,2,3,4-tetracarboxylic,
3	L-Tartaric		benzene-1,2,3-tricarboxylic
4	Phthalic	13	Benzene-1,2,3,4-tetracarboxylic
5	Citric	14	Benzenepentacarboxylic
6	Citric, glutaric	15	Mellitic
7	Glutaric	16	Mellitic,
8	Oxalic		benzene-1,2,4,5-tetracarboxylic,
9	Oxalic, succinic		pyruvic
10	Succinic	17	Benzene-1,2,4,5-tetracarboxylic
11	Benzene-1,2,3-tricarboxylic	18	Pyruvic

Since R_F values were observed to be independent of the direction of irrigation, it was possible to develop a useful two-dimensional method of paper chromatography. In this a basic solvent (without incorporated indicator) was used in the first direction followed by one of the acidic solvents in the second direction.

TABLE I

R_F VALUES OF ORGANIC ACIDS

Acid	R_F *values* (× *100*)					
	Solvent 1	*Solvent 2*	*Solvent 3*	*Solvent 4*	*Solvent 5*	*Solvent 6*
Oxalic	64–74	75–87	30–40, 49–68	0–28	0–3	3–5
Malonic	77–82	86–91	67–79	26–34	11–24	53–63
Succinic	78–89	85–92	78–86	37–49	20–37	58–70
Glutaric	83–90	90–94	83–91	40–54	25–41	62–73
Tartronic	51–68	62–75	53–63	20–30	11–21	48–64
L-Tartaric	43–53	58–68	38–46, 47–52	24–35	8–19	50–61
Citric	56–61	63–72	52–60	11–26	1–10	41–52
Pyruvic	88–95	90–96	67–87	18–50	10–39	53–78
Benzoic	98–100	98–100	98–100	72–80	68–78	79–88
Phthalic	94–97	96–100	91–96	40–51	30–39	64–72
Benzene-						
1,2,3-tricarboxylic	65–79	72–82	65–74	8–26	8–17	42–58
1,3,5-tricarboxylic	53–86	80–90	74–90	20–36	10–19	48–60
1,2,3,4-tetracarboxylic	38–56	54–69	50–64	0–18	0–5	25–38
1,2,4,5-tetracarboxylic	46–66	64–78	49–64	3–17	0–8	35–47
Benzenepentacarboxylic	32–51	45–59	20–32, 33–44, 50–64	0–6	0–3	10–21
Mellitic	17–34	32–49	12–19, 21–30, 46–54	0–3	0–3	0–3

ACKNOWLEDGEMENT

Grateful acknowledgement is made to Professor S. G. WARD of the Department of Mining and Minerals Engineering, and to Dr. L. HORTON of the Tropical Products Institute, for their continued interest and helpful advice.

SUMMARY

Rapid reliable methods have been developed for the separation of mixtures of organic acids using one-dimensional and two-dimensional techniques of paper chromatography. New solvent mixtures are proposed which include acidic, basic and buffered types. These methods, which have been proved by application to some eighty well-known organic acids, offer considerable advantages over existing techniques in their ease of operation and ready reproducibility of results.

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CHROMATOGRAPHIE PAR ADSORPTION SUR PAPIER

IV. L'ADSORPTION SUR CELLULOSE DE QUELQUES COMPLEXES MÉTALLIQUES AVEC LA THIOURÉE

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(Reçu le 9 avril 1960)

Au cours de travaux récents[1] nous avons pu montrer l'existence de certains analogies entre l'adsorption sur papier filtre des complexes halogénés et le mécanisme d'extraction par solvant. De ce fait nous nous sommes demandés si l'on pouvait aussi observer une adsorption avec des complexes de métaux et de ligands organiques.

Pour ces investigations il nous fallait des complexes et ligands hydrosolubles. Nous avons donc choisis pour nos travaux préliminaires comme agent complexant la thiourée, qui forme des complexes avec beaucoup des éléments de transition, ses complexes colorés de Os, Ru, Bi, Sb et Te étant connus par leur utilisation en colorimétrie[2]. Il se trouve que la complexation s'accompagne parfois aussi bien d'une réduction comme dans le cas de Au(III) et Cu(II).

Nos expériences ont révélé que les complexes avec la thiourée sont fortement adsorbés sur la cellulose, cette adsorption étant différent de celle des chlorocomplexes (et plus forte) parce que les complexes de la thiourée sont désorbés en augmentant la concentration de HCl dans le solvant (au lieu d'être adsorbés). En présence de la thiourée dans le solvant l'adsorption de quelques complexes augmente; d'autre part en son absence les complexes instables migrent toujours en taches compactes.

Puisqu'il est ainsi possible de chromatographier côté à côté l'ion métallique et le même ion mélangé avec la thiourée, on peut observer facilement le changement de l'adsorption dû à la présence de la thiourée.

EXPÉRIENCES

Les complexes ont été obtenus par action de thiourée en excès (soit solide soit en solution aqueuse à 10%) sur une solution chlorhydrique 2 *N* de l'ion métal; il est nécessaire de chauffer dans le cas des complexes inertes (Os, Ru, Pt etc.); le complexe bleu du ruthénium a été préparé à partir du chlororuthénite et le complexe rouge d'osmium à partir du tétraoxide.

La chromatographie a été réalisée par méthode ascendante sur papier Whatman No. 1.

La Fig. 1 montre les valeurs du R_F des complexes de Bi, Ru et Os, en solution aqueuse à 1% de thiourée, en fonction de la concentration en HCl.

La Fig. 2 montre les variations des valeurs du R_F en fonction de la concentration en thiourée (de 0 à 5 %), milieu: HCl 2 *N*, complexes: Os, Ru, Bi et Po.

Nous avons montré en Fig. 3 les chromatogrammes d'un grand nombre d'ions métalliques en solution, seuls d'un côté, en solution avec un excès de thiourée de l'autre. C'est dans le cas du Pb(II) que l'on observe le moindre écart, mais la différence est encore bien nette. La Fig. 4 montre les chromatogrammes de Po(IV), sur la même bande, côte à côte, la solution simple et la solution contenant quelques cristaux de thiourée par goutte. La Fig. 4d montre le Po(IV) chromatographié en milieu HCl 2 *N* avec 1 % de thiourée. La Fig. 5 présente des expériences similaires réalisées avec Po(IV) et d'autres agents complexants. Pas de différences de vitesses, ni avec l'acide rubéanique, ni avec l'acide rhodizonique. La diéthyldithiocarbamate produit un complexe insoluble qui reste au point d'application. Le Tableau I montre les valeurs du R_F d'un grand nombre de complexes thiourée–métaux en solution HCl 2 *N* ou encore HCl 2 *N*–butanol. D'une façon générale, il semble que les complexes stables ont des valeurs de R_F assez basses (0.2 à 0.3 en milieu HCl 2 *N* et 0 à 0.2 en milieu

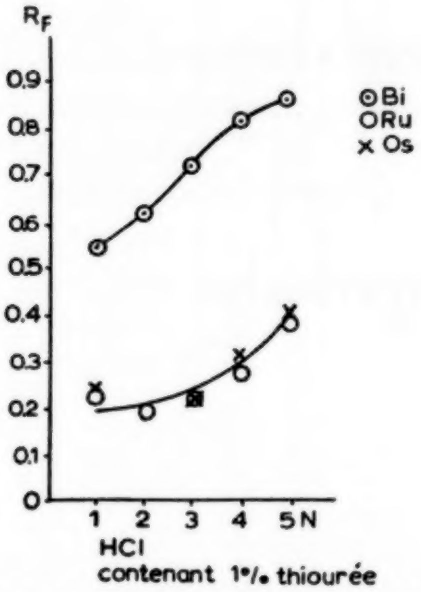


Fig. 1. Variation de valeurs de R_F avec la concentration de HCl.

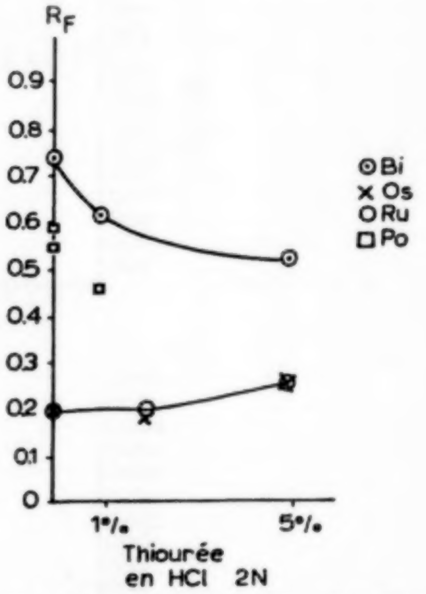
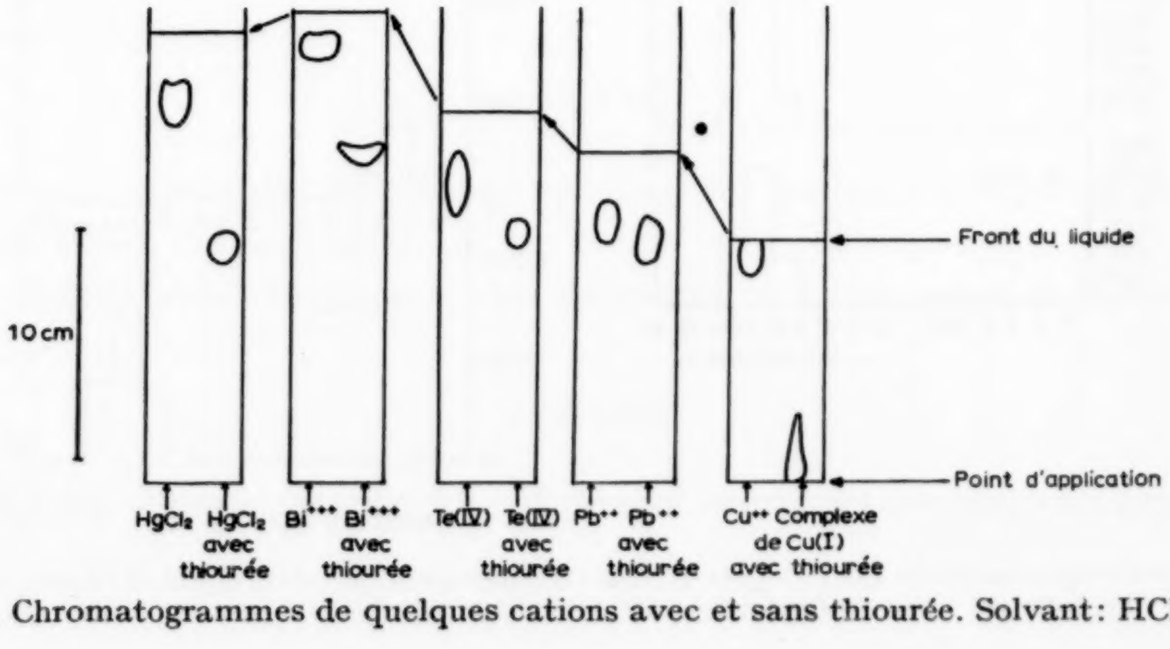


Fig. 2. Variation de valeurs de R_F avec la concentration de thiourée.



10 cm
Front du liquide
Point d'application
$HgCl_2$
$HgCl_2$ avec thiourée
Bi^{+++}
Bi^{+++} avec thiourée
Te(IV)
Te(IV) avec thiourée
Pb^{++}
Pb^{++} avec thiourée
Cu^{++}
Complexe de Cu(I) avec thiourée

Fig. 3. Chromatogrammes de quelques cations avec et sans thiourée. Solvant: HCl 2 *N*.

butanol–HCl 2 *N*); par contre les complexes instables offrent des valeurs plus élevées mais qui restent cependant inférieures à celles de l'ion non complexé correspondant.

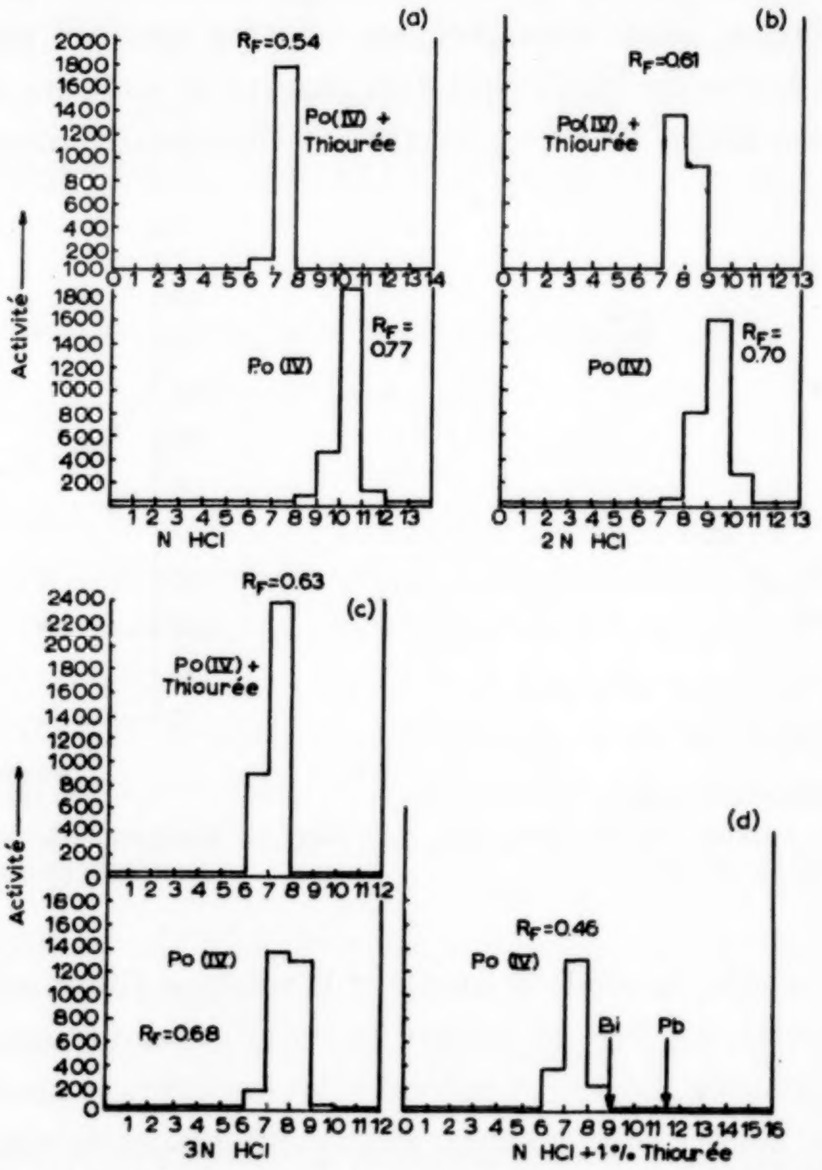


Fig. 4. Chromatogrammes du Po(IV) avec et sans thiourée. (a) solvant: HCl *N*; (b) solvant: HCl 2 *N*; (c) solvant: HCl 3 *N*; (d) solvant: HCl *N* contenant 1 % de thiourée.

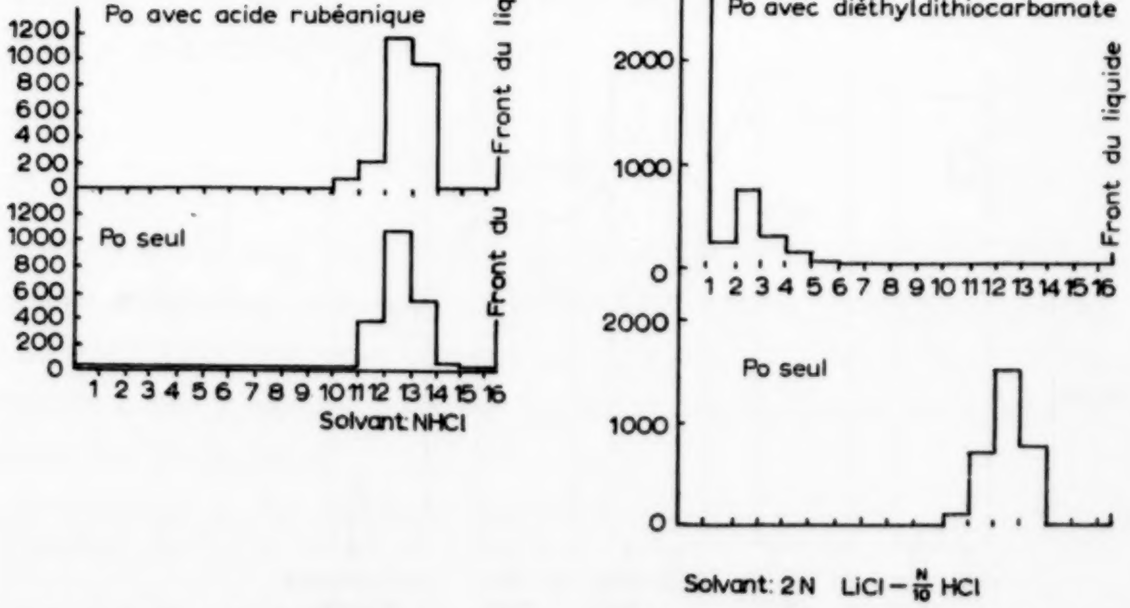


Fig. 5. Chromatogrammes du Po(IV) avec et sans complexants: (a) avec acide rubéanique, solvant: HCl *N*; (b) avec diéthyldithiocarbamate, solvant: LiCl 2 *N*–HCl 0.1 *N*.

TABLEAU I

VALEURS DE R_F DE QUELQUES COMPLEXES AVEC LA THIOURÉE

	Couleur du complexe	Solvant	
		HCl 2 N	Butanol–HCl 2 N
Pd(II)	jaune	0.32	0.12
Pt(IV)	jaune	0.33	
Au(I)	incolore	0.34	0.60
Rh(III) (préparé à partir de $RhCl_6^{3-}$)	orange	0.20	0.03
Ru(III) ?	bleu	0.20	0.08
Os (à partir de OsO_4)	rouge	0.20	0.05
Bi(III)	jaune	0.74	0.61
Sb(III)	jaune	0.63	comète 1.0
Re(IV)	jaune	0.21	0.02
Tc(IV)	orange	0.21	0.03

DISCUSSION

L'adsorption chromatographique de complexes avec la thiourée en milieu acide relativement concentré offre de nombreuses et intéressantes perspectives. On a déjà noté[3] que la découverte de nouveaux complexes est presque toujours due soit à la formation d'un précipité soit à une solution colorée, mais cette découverte est rarement le fruit de mesures de constantes de stabilité. Il en est de même, et pour cause, avec les radioéléments disponibles seulement en microquantités. La comparaison du mouvement avec et sans complexant, bien qu'elle ne soit pas toujours concluante peut permettre la détection de réactions de complexation. Nous avons montré que le polonium, dont le complexe avec la thiourée était inconnu, est adsorbé plus fortement en son présence et que sa valeur de R_F varie avec la concentration en thiourée.

Cette adsorption nous semble dû à une complexation, peut-être accompagnée par un changement de valence. Il est improbable qu'il provienne d'une réduction sans complexation puisque le polonium ne change pas sa valeur de R_F en présence de réductants tel que le SO_2.

RÉSUMÉ

L'adsorption chromatographique sur cellulose de quelques complexes métalliques avec la thiourée a été investigée.

Les observations indiquent qu'un complexe se forme entre le polonium et la thiourée.

SUMMARY

The adsorption chromatography on cellulose of some metal–thiourea complexes was studied.

The findings indicate that polonium forms a complex with thiourea.

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Short Communications

Analysis of phosphorus trichloride and oxychloride mixtures

Gas phase chromatography of organic materials, especially hydrocarbons, is already a well established analytical technique but is not often applied to the separation of inorganic compounds.

During the last two years we have analysed volatile inorganic compounds, such as acid chlorides, by gas–liquid chromatography while determining the purity of radioactive labelled compounds.

Conversations with other workers show a growing interest in this field, so the following conditions for separating phosphorus trichloride and oxychloride may be of value.

Equipment consists of a Griffin and George Mk. II Gas Chromatograph with katharometer detection and sample injection by a weighed capillary[1].

Column. 6 ft. × ¼ in. 23 % w/w silicone elastomer E301 on 80–120 mesh celite 545.

Temperature. 63°.

Carrier gas. 1.75 l of nitrogen per hour, pre-dried in a 3 ft. tube of anhydrone.

Inlet pressure. 63 cm.

Outlet pressure. 24 cm.

Bridge current. 100 mA.

Detector sensitivity set × ¼ for stability.

Response is linear for both compounds using 1 to 10 mg samples, over 10 mg giving an off scale recorder deflection.

By injecting *ca.* 100 mg sample, down to 0.25 % w/w of the minor component may be determined and at this sample level the katharometer remains stable. A sample of commercial B.D.H. phosphorus trichloride contained 1.5 w/w of oxychloride. Retention times measured from the injection pressure change are 8.5 and 15.5 minutes for the trichloride and oxychloride respectively.

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Relative detector response in gas chromatography
III. Aliphatic esters

The investigation of the relative detector response of a thermal conductivity detector to organic compounds of various types when nitrogen is the carrier gas is continued with a study of the relative responses to aliphatic esters. MESSNER *et al.*[1], using helium as the carrier gas, found that the relative responses to aliphatic esters increased with increasing molecular weight.

The object of the present work was to investigate the relative detector responses to aliphatic esters to find

(a) if isomeric esters had similar responses,

(b) if the effect of chain branching was similar to that found previously in the alkylbenzenes[2], and

(c) the effect of various substituent groups on the relative responses.

Experimental. Gas-liquid chromatography was carried out using the apparatus described previously[3]. Nitrogen was used as the carrier gas at a flow rate of approximately 33 ml/min, and the bridge current was 100 mA. Under the conditions used no reversal of peaks was observed for any of the compounds studied.

2-Chloroethyl formate was prepared by the chlorination of 1,3-dioxolan[4]. 2-Acetoxyethanol was separated from the diacetoxy compound using a preparative gas chromatograph. The other esters were prepared and purified using standard methods.

Results and discussion. The relative detector responses to a number of aliphatic esters are shown in Table I.

An examination of these results shows that there is an increase in relative response with increasing molecular weight. Also, isomeric esters have similar relative responses provided that there is no chain branching. It is found that there is a decrease in relative response to isomeric esters with an increase in chain branching: this decrease in relative response is similar to that found for alkylbenzenes[2].

The relative detector responses to unsaturated aliphatic esters are similar whether the unsaturation is in the alcohol component or in the acid component and these relative responses are lower than those expected from saturated esters of similar molecular weights.

The relative detector responses to a number of substituted aliphatic esters are shown in Table II. In each homologous series there is a regular increase in relative response with increasing molecular weight. It is also found that the position of a substituent group has a large effect on the relative response. A compound with a substituent group occupying a position adjacent to the ester group has a lower relative response than the isomeric compound with the substituent occupying a position farther along the chain; this effect is much less marked in the alkoxy esters. This positional effect is somewhat similar in magnitude to the *ortho*-effect reported previously[2].

TABLE I

RELATIVE DETECTOR RESPONSE TO ALIPHATIC ESTERS

Compound	*Mol. wt.*	*Response per mole relative to benzene (= 100)*
Saturated esters		
Methyl formate	60	70
Ethyl formate	74	92
Methyl acetate		98
n-Propyl formate	88	114
Isopropyl formate		107
Ethyl acetate		119
Methyl propionate		114
n-Butyl formate	102	135
Isobutyl formate		130
sec.-Butyl formate		126
n-Propyl acetate		137
Isopropyl acetate		133
Ethyl propionate		133
Methyl *n*-butyrate		135
Methyl isobutyrate		130
n-Butyl acetate	116	158
Isobutyl acetate		157
sec.-Butyl acetate		146
tert.-Butyl acetate		139
n-Propyl propionate		157
Isopropyl propionate		149
Ethyl *n*-butyrate		153
Ethyl isobutyrate		149
Methyl *n*-valerate		158
n-Amyl acetate	130	178
n-Butyl propionate		174
Isobutyl propionate		172
sec.-Butyl propionate		168
n-Propyl *n*-butyrate		174
Isopropyl *n*-butyrate		169
n-Propyl isobutyrate		171
Isopropyl isobutyrate		164
Ethyl *n*-valerate		178
Methyl *n*-hexoate		178
n-Butyl *n*-butyrate	144	196
Isobutyl *n*-butyrate		192
sec.-Butyl *n*-butyrate		186
n-Butyl isobutyrate		187
Isobutyl isobutyrate		183
sec.-Butyl isobutyrate		173
n-Hexyl acetate		199
Methyl *n*-heptoate		200
Unsaturated esters		
Allyl formate	86	105
Allyl acetate	100	128
Methyl crotonate		127
Allyl propionate	114	148
Ethyl crotonate		149
Allyl *n*-butyrate	128	168
n-Propyl crotonate		171
Isopropyl crotonate		163

TABLE II

RELATIVE DETECTOR RESPONSE TO SUBSTITUTED ALIPHATIC ESTERS

Compound	*Mol. wt.*	*Response per mole relative to benzene (= 100)*
Chloro esters		
2-Chloroethyl formate	108.6	139
Methyl chloroacetate		141
Ethyl chloroacetate	122.6	166
Methyl 1-chloropropionate		152
Methyl 2-chloropropionate		168
n-Propyl chloroacetate	136.6	188
Isopropyl chloroacetate		173
Ethyl 1-chloropropionate		170
Ethyl 2-chloropropionate		191
Hydroxy esters		
Methyl glycollate	90	73
Ethyl glycollate	104	96
Methyl lactate		83
2-Acetoxyethanol		100
Ethyl lactate	118	108
Alkoxy esters		
Methyl methoxyacetate	104	122
Ethyl methoxyacetate	118	146
Methyl ethoxyacetate		148
2-Methoxyethyl acetate		152
Ethyl ethoxyacetate	132	170
2-Ethoxyethyl acetate		176
Keto esters		
Methyl pyruvate	102	101
Ethyl pyruvate	116	122
Methyl acetoacetate		144
Ethyl acetoacetate	130	165

The nature of the substituent group also has an effect on the relative response. Hydroxy, alkoxy and keto substituted aliphatic esters have lower relative responses than those expected from unsubstituted esters with similar molecular weights. A chloro substituent has not such a marked effect, methyl chloroacetate has a relative response slightly lower than an unsubstituted ester of similar molecular weight and both ethyl chloroacetate and *n*-propyl chloroacetate have relative responses slightly higher than unsubstituted esters having similar molecular weights.

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Chromatographic separation of alkali metal ions on paper impregnated with ammonium molybdophosphate

In view of the difficulty of obtaining satisfactory separations of alkali metal ions by paper chromatography[1-12], the possibility was examined of separating these elements by means of ascending chromatography with ion-exchange strips along the lines described in an earlier paper[13].

Ammonium molybdophosphate (AMP) was chosen as the cation exchanger since this inorganic exchanger has a separation factor for pairs of alkali metals that is more than ten times as high as that of Dowex 50[14-15].

Preparation of the ion-exchange paper

6 g of AMP, prepared by the direct precipitation method described by SMIT *et al.*[15], are dissolved in 25 ml NH_4OH (1:4). The solution is then placed in a Petri dish (10 cm diam.) and ten strips of Whatman No. 1 filter paper (35 × 4 cm) are drawn slowly through it. The strips are then hung up to dry at room temperature.

Following this, each strip is placed in turn for 10 min in a special glass container (50 × 12 × 12 cm) with a solution of 25 g ammonium paramolybdate, 104 ml 65 % nitric acid and 638 ml distilled water to obtain a second precipitation of the AMP in the strips. In order to maintain the solution at constant concentration, it is advisable after treating every three strips to add 1 ml 65 % HNO_3 containing 33 mg ammonium paramolybdate.

The strips are once more hung up to dry. They are then washed first in a solution of 1 N ammonium nitrate for 10 min and then twice (for 30 min each time) in distilled water, in order to remove all traces of the ammonium nitrate.

If the strips are required for separating sodium from other alkali metals, it is important after the ammonium nitrate washing, that the strips should be handled carefully in order to avoid any contamination.

Experimental

Strips of Whatman No. 1 filter paper, impregnated with 3.2 mg/cm^2 AMP, were used to determine the R_F values of the alkali metal ions.

The solutions of metal ions were prepared by dissolving the nitrates. The chromatograms were developed by the ascending method for 2–3 h.

The R_F values obtained under varying conditions of elution are given in Table I. From these values it can be seen that, with a suitable developing solvent, it is possible to obtain clear separations of any two ions.

The difficulty in obtaining a separation of the 5 alkali metals on the same strip at the same time with a single eluant is due to the considerable difference in the affinity of the AMP for the elements in question. Thus, for example, while Li^+ and Na^+ ions move more or less with the liquid front in dilute solutions, such as 0.1 M NH_4NO_3, Cs^+ and Rb^+ remain at the point of application even when more highly concentrated solutions (*e.g.* 0.5 M NH_4NO_3) are employed as eluant.

TABLE I

R_F VALUES OF ALKALI METAL IONS IN VARIOUS SOLVENTS

Element	H_2O	0.1 N $HClO_4$	0.1 N HNO_3	0.1 N HNO_3 0.2M NH_4NO_3	0.1N HNO_3 0.3M NH_4NO_3	0.1N HNO_3 0.5M NH_4NO_3	0.2N HNO_3 3.5M NH_4NO_3	10M NH_4NO_3
Li	0.83	0.80	0.78	0.78	0.79	0.81	0.90	0.91
Na	(0.38) diffuse	0.74	0.73	0.77	0.77	0.80	0.89	0.90
K	(0.08) diffuse	0.32	0.27	0.56	0.60	0.69	0.86	0.90
Rb	0	0.03	0.04	0.06	0.06	0.07	0.60	0.80
Cs	0	0	0	0	0	0	0.10	0.14

Zinc uranyl acetate is employed for detecting Na^+ and Li^+ ions. They give a bright greenish yellow fluorescence in U.V. light. Potassium is detected by flame photometry. Caesium and rubidium are detected through their radioactive isotopes ^{137}Cs and ^{86}Rb. A 10% equivalent solution of $AuCl_3$ and $PdCl_2$ is also employed for detecting Cs^+ ion.

In order to obtain a complete separation of the 5 alkali metals we, therefore, advise the following procedure:

A strip (45 × 4 cm) of Whatman No. 1 filter paper, impregnated with AMP as described above, is spotted with a mixture of the 5 alkali metals and developed for 32 cm with a solution of 0.1 *N* HNO_3 and 0.2 *N* NH_4NO_3. With this eluant, Cs^+ and Rb^+ ions stay at the point of application, Na^+ and Li^+ ions move a little below the liquid front, while K^+ moves with an R_F of 0.56.

The exact position of the potassium is calculated from the R_F value and the strip

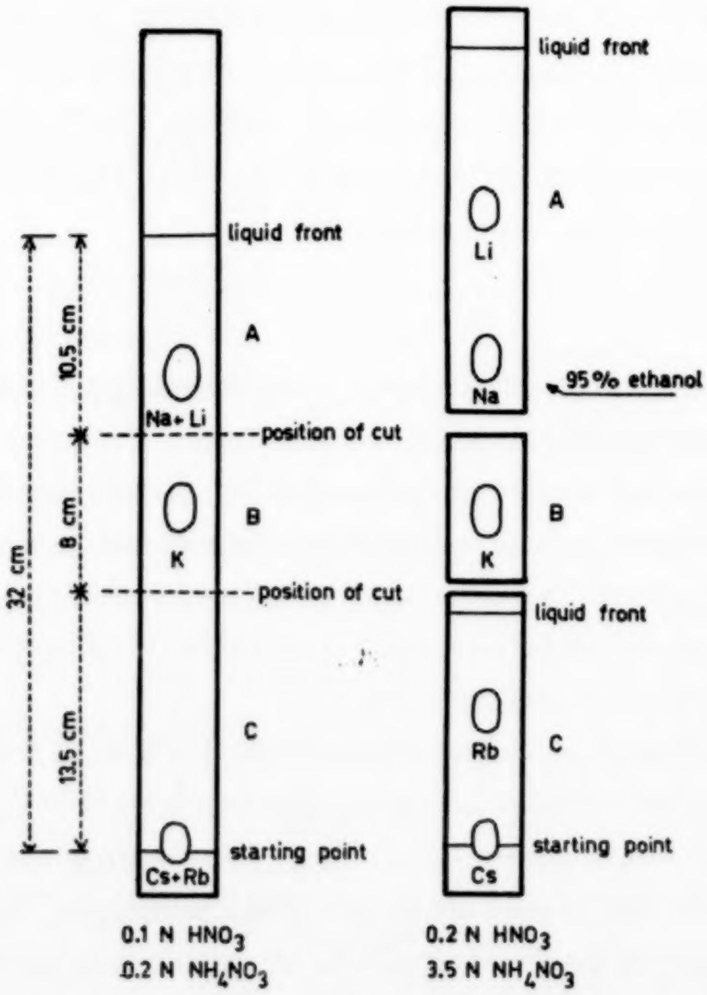
liquid front
A
10.5 cm
Na+Li
position of cut
8 cm
B
K
position of cut
32 cm
C
13.5 cm
starting point
Cs+Rb
0.1 N HNO3
0.2 N NH4NO3
liquid front
A
Li
Na
95% ethanol
B
K
liquid front
C
Rb
starting point
Cs
0.2 N HNO3
3.5 N NH4NO3

Fig. 1. Separation of alkali metal ions on AMP-impregnated paper.

is then cut, as indicated in Fig. 1, in such a way that it is divided into three parts, *A*, *B*, and *C*. On *A* lithium and sodium are found, on *B* potassium and on *C* caesium and rubidium.

In order to separate sodium and lithium, the strip is eluted with 95 % ethanol. Sodium remains at the point of origin and lithium moves with an R_F of 0.55.

On strip *C*, Cs^+ and Rb^+ ions are readily separated with a 0.1 *N* HNO_3 and 3 *N* NH_4NO_3 solution, only a short development being required.

Fig. 1 shows the separation thus obtained of 15 γ of Na^+ and K^+, and 100 γ of Li^+, Rb^+ and Cs^+ ions.

Acknowledgements

The authors wish to thank Prof. V. CAGLIOTI and Dr. M. LEDERER for their kind advice and Mr. P. D'AMARIO for his valuable help.

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Chromatographic separation of inorganic ions on glass fiber strips using fused salts as eluant

The aim of the present research is to examine the possibility of separating various ions on glass fiber strips by means of ascending chromatography with fused salts as eluant.

Chromatographic separations of a number of transition metals have been achieved by GRUEN *et al.*[1] and by BENARIE[2], on γAl_2O_3 and on Pyrex glass powder columns respectively, whereby fused salts were used as eluant.

Glass fiber paper (Whatman G.B., 0.65 mm thick; weight: 150 g/m²) was pretreated with HNO_3 1:1 and washed three times with distilled water. $LiNO_3$–KNO_3 eutectic was chosen as the fused eluant (43 mol. % $LiNO_3$, m.p. 132°), with and without the addition of KCl as complexing agent.

The experiments were carried out at 160° ± 2° in Pyrex glass cylinders with the fused salts placed on the bottom. Under these conditions Pb (II), Cd(II), Ba*(II), Ca*(II), Sr*(II), Rb*(I) and Cs*(I)* move with the liquid front in the fused salts, while Fe(III), Ag(I), Th(IV) and U(VI) remain at the point of application. Cu(II), Co(II) and Ni(II), on the other hand, form long tails.

In the elutions carried out with $LiNO_3$–KNO_3 eutectic with added KCl, it was observed that Fe(III), Cu(II), Co(II) and Ag(I) reacted rather markedly with the Cl^- ions, and in all cases shifted towards the front as the Cl^- ion concentration in the fused eutectic increased.

We further examined the possibility of carrying out ion exchange chromatography on zirconium phosphate (ZP)-impregnated glass fiber strips, along the lines described in an earlier paper[3].

In view of the high eluting power of the fused eutectic due to the strong concentration of K^+ and Li^+ ions, the ZP had an ion exchange retarding effect only in the case of such ions as Cs^+ and Rb^+ for which, as is well known, it has great affinity.

In the case of other ions only slight differences were observed between the results on ZP-impregnated strips and on those not so impregnated.

In all probability, other ion exchange separations are possible with eluants that are only slightly ionized.

We are at present studying the chromatographic separations mentioned above, at different temperatures. Details and further results will be published in due course.

Acknowledgements

The authors express their thanks to Prof. V. CAGLIOTI and Dr. M. LEDERER for their kind advice.

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* In view of the difficulty of revealing alkali and alkaline earth ions by means of chromatic reactions, radioactive isotopes, marked*, were employed.

BOOK REVIEWS

Gas Chromatography Abstracts 1958 (references to the end of 1958), compiled and edited by C. E. H. KNAPMAN, Assistant Editor C. G. SCOTT, Butterworths Scientific Publications, London, 1960, 262 pages, price 42 s.

This work was compiled from three independent bibliographies of members of the Gas Chromatography Discussion Group. It is intended to serve as an inaugural volume of *Gas Chromatography Abstracts* which is planned to appear annually.

The volume under review consists of the bibliography up to the end of 1958 and contains 1468 references with the complete title of the paper, the reference, its reference in *Chemical Abstracts*, as well as a short paragraph describing its contents.

The number of references appears rather large at first sight when compared with only 619 references in the review by HARDY AND POLLARD (this journal, Vol. 2 (1959) 1) for almost the same period. However, not only gas–liquid chromatography is abstracted here but also gas adsorption chromatography and numerous papers dealing with chromatography, gas adsorption or gas analysis in general. The editors are to be congratulated for compiling these abstracts. In a field such as gas chromatography, in which about five hundred papers are published annually, text books and review articles will always need such annual volumes as supplement. Such a compilation is also justified although it duplicates in a sense the work of *Chemical Abstracts* and *Analytical Abstracts*, since most information can be gained here by consulting only one subject index, which incidentally is very well prepared.

The reviewer found very few printer's errors, and no important references seemed to be missing.

Papierchromatographie in der Botanik, 2nd Ed., edited by H. F. LINSKENS, published by Springer-Verlag, Berlin, Göttingen, Heidelberg, 1959, 408 pages, 124 figs., 2 coloured plates, price D.M. 58.—.

This second edition reflects great credit on the editor and his 23 co-authors. The first edition, of 1955, has been expanded from 243 to 394 pages, and the number of contributors has been increased from 13 to 23, the present authors including no less than 11 of the original ones.

In the introductory section a brief but very clear exposition of the theory of paper chromatographic separations is followed by excellent sections on laboratory equipment and techniques, with many illustrations and practical instructions. Sources of error, types of paper, the pretreatment of papers, the extraction and preparation of samples from plant materials, the removal of interfering substances, the applications of isotope techniques are all reviewed fairly fully, always with emphasis on the practical aspects. Throughout the book, paper electrophoresis is given as important a place as paper chromatography.

The remainder of the book comprises chapters on the following special topics: Inorganic anions and cations; Carbohydrates; Organic acids; Lichen acids; Phosphatides and complex lipids; Proteins and their building blocks; Nucleic acids and their building blocks; Plant viruses; Pigments; Growth substances and related compounds, and vitamins; Antibiotics and toxins; Aldehydes and ketones; Phenols and tannins; Organic bases, including alkaloids; Sterols and related compounds; Detection of steroids by colour reactions.

There is remarkable consistency in the high quality, the comprehensiveness and the layout of these chapters. In most of them, methods of extraction and preparation for chromatography of the substances in question are reviewed critically, chromatographic or electrophoretic separations, methods of detection of spots and quantitative determinations are discussed, and comprehensive tables of R_F values are given. For biologically active compounds, bioassay methods and their application to spots on chromatograms, or eluates of these, are discussed. The many references to the literature appear to be well chosen.

One feels that more cross-references would be helpful. For example, desalting is mentioned briefly on p. 6, but there is no reference there to the valuable discussion of apparatus and techniques which appears on pp. 32–34, or of their application to amino acids on pp. 150–151. Continuous (curtain) electrophoresis is not treated in detail in the introductory section, though references to the use of this important technique of course appear later—in the chapter on plant viruses, for example. For staining peptide spots on paper, ninhydrin only is mentioned; the chlorine–starch iodide method of RYDON AND SMITH (*Nature*, 169 (1952) 922) is more sensitive as a general stain, and of course other stains are invaluable for particular groups of peptides.

The reviewer feels that in addition to providing a valuable handbook on the paper-chromatographic separation of plant materials, this book will furnish a useful supplement to existing works on the extraction and isolation of plant constituents.

J. E. FALK (Canberra)

NEW BOOKS

Gas Chromatography Abstracts 1959, edited by C. E. H. KNAPMAN AND C. G. SCOTT, Butterworths Scientific Publications, London, 1960, viii + 164 pages, price 42s, by post 1s 6d extra.

The Thyroid Gland, Medical Department, The British Council, London, 1960, 85 pages, price 20 s.
Volume 16, No. 2 of the *British Medical Bulletin*.

Quantitative Paper Chromatography of Steroids, edited by D. ABELSON AND R. V. BROOKS, Cambridge University Press, London, 1960, 103 pages, price 30 s net.
Memoirs of the Society for Endocrinology No. 8.

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SOME COMMENTS ON THE CONSTRUCTION AND OPERATION OF THE GAS DENSITY BALANCE

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(Received March 18th, 1960)

In the past three or four years a great deal of effort has been devoted to the development of new types of detector for use on vapour-phase chromatographic equipment. Emphasis has been heavily in the direction of ever-increasing sensitivity, and newcomers to the field might be forgiven for allowing themselves to become persuaded that no detecting system which does not give a full-scale reading with 10^{-10} g of a carefully chosen compound is worth considering. The principal purpose of this paper is to draw attention once again to one of the first detectors to be developed for the purpose, the gas density meter or balance of MARTIN AND JAMES[1]. This instrument has many merits. In use it is robust and reliable (in spite of remarks in the literature to the contrary sense). It does not require elaborate ancillary equipment. It is linear over a wide range in its response to the concentration of sample vapour in the carrier gas and although the mass sensitivity varies with the molecular weights of samples and carrier gas the relationship is a simple one, and it is otherwise independent of chemical differences. The sample is not pyrolysed or affected in any way by the instrument, and collection from the effluent gas presents no particular difficulties. The sensitivity is as high as many normal problems are likely to require, and with fairly simple amplification and recording equipment it corresponds quite conveniently with the minimum quantities readily measurable by other physical means (including ultraviolet spectroscopy), *e.g.* a sample of 0.1 mg will give a peak whose area is reproducible to perhaps ± 2% if the retention volume is within reasonable limits. This range of qualities cannot be paralleled by any other available detecting system.

The lack of interest in the gas density balance in practice is a consequence of the difficulty of making it. The design is ill-suited to mass production and it is therefore not fitted as a standard detector to any commercial gas chromatographic assembly. The difficulties of construction have to be admitted, but patience rather than any special technical skill is required, except perhaps for the final sealing-in of the plugs blocking the drill-holes. Various alternatives to the drilled copper block have been tried, including tubular skeleton designs either left as such[2] or cast afterwards into a metal block[3], and also a block built up from slabs of copper in which the channels which could be arranged in suitable lengthwise planes were milled out and the others drilled[4]*. In all these designs the same problem has ultimately to be faced, that of ensuring completely gas-tight joints without running any risk of occluding channels. When we were considering the construction of two new gas density balances we felt that none of these alternatives had advantages great enough to warrant departure from the general design described by MARTIN AND JAMES. A number of detail modifications were, however, made, principally as a result of several years' experience by Dr.

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* Exhibited at the Physical Society Exhibition, London, 1960.

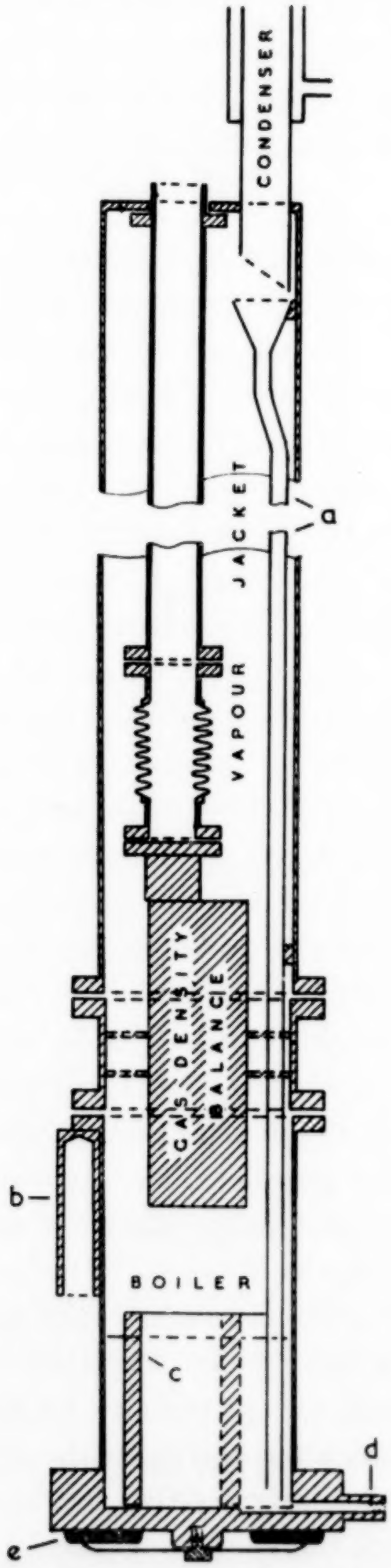

Fig. 1. Vapour-phase chromatography equipment. The column seating boss on top of the gas density balance, the inner vapour jacket, condenser and liquid return tube do not all lie on the same longitudinal cross-section as that represented by the lower part of the diagram, but for the sake of clarity they are drawn "solid". a – liquid return tube; b – socket for thermal cut-out switch; c – splash baffle (on three legs); d – nitrogen inlet and drain tube; e – heater element.

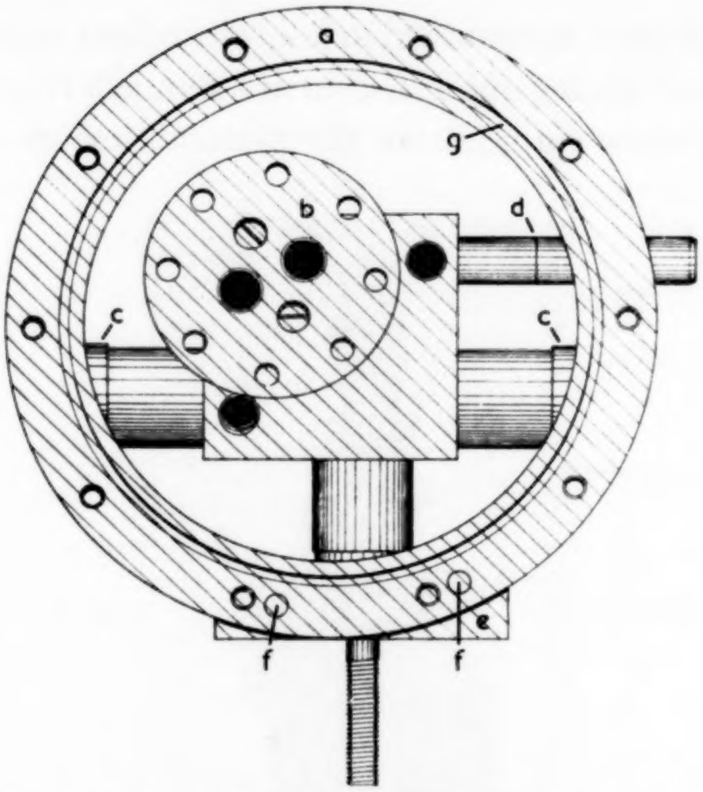

Fig. 2. Top view of gas density balance mounted in its flanged ring. a – outer vapour jacket flange; b – inner vapour jacket flange; c – collars on thermocouple support tubes; d – join in gas exit tube; e – supporting block for anemometer heater; f – pins for securing (e); g – groove for gasket. Features (c) and (d) are necessary for assembly. Without them it would not be possible to fit the balance block, complete with side tubes, into the flanged ring.

A. T. JAMES and the present authors with the original models in use in Dr. JAMES's laboratory. The most important defect of these proved to be the permanently sealed design of the vapour jacket and the consequent difficulty of dismantling the equipment when, as inevitably happens, some unforseen accident required it. It was therefore decided to fit the new instruments with flanges to connect them with the boiler and with the outer and inner vapour jackets, which themselves then required a further flange to join them at the top (Fig. 1). Lead wire gaskets were used initially, but graphited asbestos gaskets have proved more satisfactory. The whole assembly may

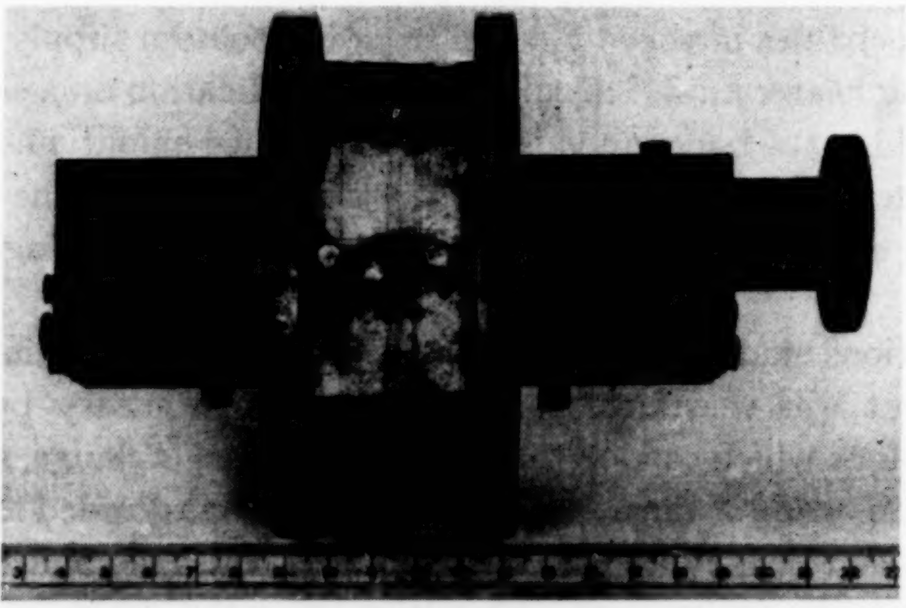

Fig. 3. Front view of gas density balance mounted in its flanged ring, showing the supporting block for the anemometer heater. The two bolts projecting from it are intended to hold the heater insert in position as described by MARTIN AND JAMES. The numerous lugs projecting from the balance are the ends of the copper plugs closing the drilled channels.

conveniently be covered with a standard size of fibreglass rigid-section pipe lagging. Figs. 2 and 3 show the balance mounted in its flanged ring in top and side view respectively, and Fig. 4 shows in addition the boiler, with one of the two steel pivots about which the entire column assembly may be tilted, and also the bellows coupling used at the bottom end of the inner vapour jacket to give a little flexibility when tightening up the flanges. The cold reflux from the condenser is returned down a separate inner tube, and the drainage tube at the bottom of the boiler is fitted with a drilled plug which enables nitrogen bled off from the column supply to bubble slowly through the boiling heater fluid. This gives a direct indication on a simple manometer of the depth of fluid in the boiler and also serves to reduce oxidation. A thermal switch is also fitted to the boiler casing to cut off the power supply if the boiler overheats, *i.e.* boils dry, and a relay prevents the supply being switched on again when it cools down.

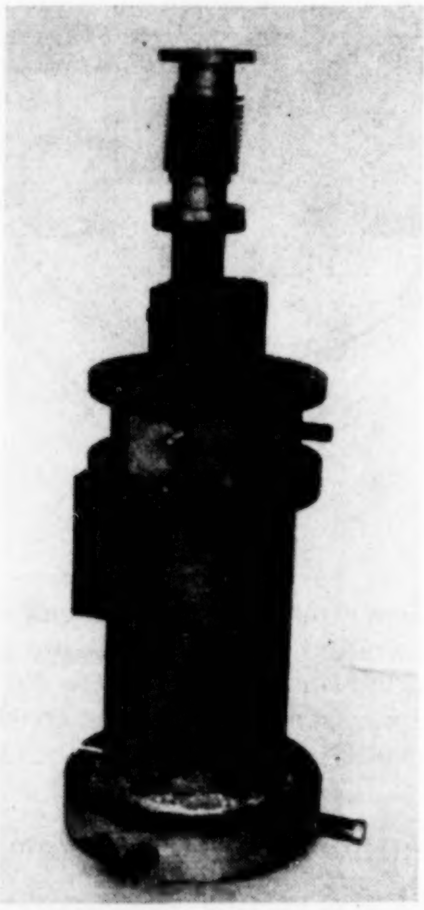

Fig. 4. The balance in position on the boiler unit. At the bottom may be seen (left of centre) one of the two pivots about which the apparatus may be tilted, and (on the right) the drain tube, closed in the photograph by a plain plug; in use it carries a nitrogen inlet tube. At the top is shown the flexible coupling which forms part of the inner vapour jacket.

The modifications made to the balance itself were essentially minor ones, although the internal layout was rearranged. This was made necessary by the cylindrical extension to the block which carried the inner vapour jacket flange, since this covered the original position of one of the upper control rods. It was decided to retain the upper control rods only, so that any adjustments required could be made with the balance supported by the boiler in its working position, and the design modifications were made in such a way that the narrow-bore tubes carrying these rods were somewhat shorter than their unrestricted opposite numbers. The instrument does not appear to be particularly sensitive to the position of these control rods, and following MUNDAY AND PRIMAVESI[2], they will probably be omitted altogether in any subsequent instruments made, as there is no difficulty in backing-off small out-of-balance signals electrically. In Fig. 6 an attempt has been made to show the internal arrangement of this model and it is hoped that in conjunction with the cross-section given in Fig. 5 and the diagrams in the original MARTIN AND JAMES paper it may be intelligible. The lettering of the diagrams conforms with that used in the original paper. In other respects no significant changes have been made. The anemometer heater assembly is

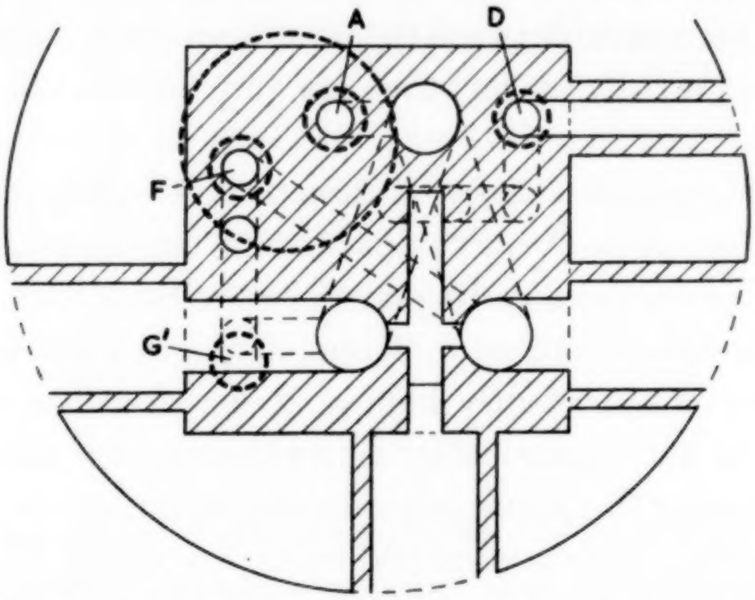

Fig. 5. Cross-section of gas density balance in the plane of the anemometer. The heavily dotted circles give the positions of the four apertures in the top of the block (lettered as in Fig. 6) and the outline of the column seating boss which carries the inner vapour jacket flange.

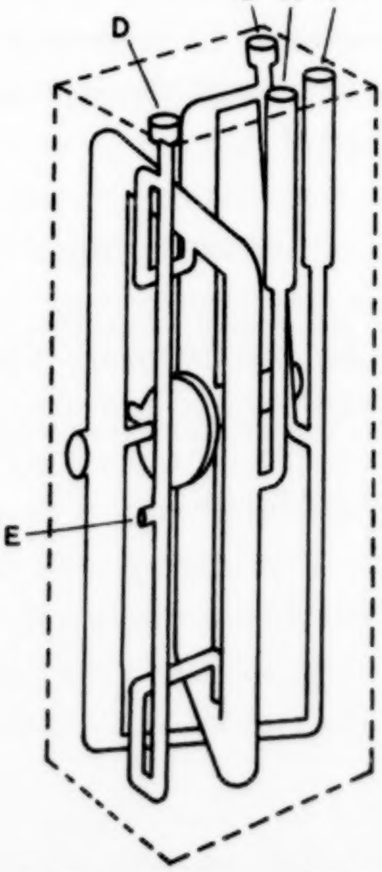

Fig. 6. Internal lay-out of gas density balance. Not to scale. A – inlet from chromatogram column; D, G' – control rod tubes (G' is tilted to avoid the thermocouple seating); E – gas exit; F – inlet from control column.

similar to the original, but the thermocouple supports are now entirely of copper clamped between two insulating washers made of any one of a number of suitable commercial gasket materials. The replacement of broken thermocouples would be accomplished more quickly if it had to be done more often since practice in this operation is a help. Thermocouples seem to last at least three months' fairly continuous operation at 200°, and usually longer; they are quite unaffected by any normal laboratory shock.

The thermocouple pair is connected directly via screened leads to a Pye D.C. Galvanometer Amplifier in two very compact units, catalogue Nos. 11341 and 11351. This is supported by a steel plate resting on a piece of thick foam rubber, and supplies an Evershed and Vignoles recording milliammeter, 2 mA full scale, 6 in. chart. The quality of the output is consistently high, and suggests that a fivefold increase in amplification would be quite practicable. Zero adjustment is carried out as previously described first by rotating the anemometer heater mounting for a coarse adjustment, and finally by tilting the entire column and balance assembly about the pivots on the boiler. A bell-battery and potentiometer network is also used to supply up to $\pm$ 100 μV at low resistance for electrical balancing when this is more convenient. Electrical pulses, either radiated or transmitted by the mains supply from unidentified sources (probably in this building), originally caused some trouble, but have been very largely damped out by a 500 μF electrolytic capacitor across the milliammeter, with no serious increase in response time.

SUMMARY

A slightly modified gas density balance is described, incorporated into vapour-phase chromatography equipment designed for simple maintenance. The newer detectors have not rendered this type of instrument obsolete since it has a range of properties possessed by no other single detector.

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A TWO-RECORDER INTEGRATOR-READOUT SYSTEM FOR GAS–LIQUID CHROMATOGRAPHY*

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(Received March 23rd, 1960)

INTRODUCTION

The increasing use of gas–liquid chromatography for quantitative analysis and the rapidity with which multi-component samples can be resolved and detected has created a need for an automatic integrator-readout system. In such an instrument it is desirable to automatically integrate the curves presented by the detector-strip chart recording potentiometer system and to automatically store this information by means of a digital readout system. The advantage of such an instrument, especially when used in conjunction with the detector developed by LOVELOCK[1], is that a numerical value, proportional to the amount of each component in the sample, is computed and stored as each component is resolved by the gas chromatograph. An automatic integrator-multiple readout system is of great value when large numbers of analyses are carried out or when the components are resolved during a short period of time. Another principle which can be incorporated into the above system is an expansion of the scale by the use of two recorders. To accomplish this, a second recorder plots a signal which is some fraction of the signal plotted by the first recorder. For example, a two-recorder system in which the second recorder sees one-tenth the signal seen by the first recorder approximates a chart width ten times that of a single recorder. Such a system greatly increases the range of sample size which can be analyzed in a single run. This is advantageous where the exact sample size is unknown or where the amounts of the various components in a sample vary over a wide range.

The present paper describes an automatic, two-recorder integration-readout system which utilizes the above principles, and which has given satisfactory service for over one year of operation. The apparatus is composed of two recorders, two integrators, and a register unit. Summation and storage of information sent out by the two integrators is performed by the register unit. The unit contains ten registers and the components necessary to direct, automatically and in sequence, the integrator signals to appropriate registers.

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* This work was supported, in part, by funds provided by the Bureau of Medicine and Surgery, U. S. Navy Department, and the Federal Civil Defense Administration. The opinions or assertions contained herein are the private ones of the authors and are not to be considered as official or as reflecting the views of the Navy Department.

GENERAL PRINCIPLE OF OPERATION

Following introduction of a multicomponent sample into the gas chromatograph, two recorders plot an index of the concentration of sample vapor in the detector *versus* time. One recorder plots a predetermined fraction of the scale plotted by the other. The area under each peak on each recorder is measured by integration as the data are plotted and the digital information for each peak area is stored on one of five registers available to each recorder. The signals from the integrators are shifted from one register to the next either manually or automatically. In the latter case automatic advance to the next register takes place as each peak is completed. No signal reaches the registers between peaks, thus, errors due to spurious counts when there are long periods between peaks are eliminated. Advance of the signal from one register to the next can be accomplished manually at any time, thus allowing approximate areas to be obtained for individual peaks which are incompletely resolved*. The apparatus can record five answers from each recorder and then shut off, or it can continuously record answers. In the latter case when the last register in each group of five registers has been used, the first register is automatically brought back to operating position and the sequence of five registers starts again. During automatic operation (or whenever desired) the register holding the oldest answer is cleared automatically each time advance to the next register takes place. In addition, appropriate switching is included so that all ten registers may be used either automatically or manually with either one of the two recorders.

DETAILS OF CONSTRUCTION AND OPERATION

Two strip chart recorders** receive the signal from the detector-impedance convertor system*** as shown in Fig. 1. The signal to the second recorder is attenuated with respect to the first recorder by means of appropriate resistances (S22)†. Base line adjustment of the second recorder is accomplished by a suitable e.m.f. applied to the recorder††. Since the potentiometer recorders produce a back e.m.f. when they are driven off scale, switching must be included so that when the first recorder goes off scale, the signal to this recorder is disconnected (S19). When the signal has decreased to a value within the range of the disconnected recorder the second recorder operates a switch (S20) which reconnects the signal to the previously disconnected recorder.

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[*] The sum of the areas will be exactly equal to the total area of the incompletely resolved components.

[**] Brown Electronik recorders, manufactured by the Minneapolis-Honeywell Regulator Co., Philadelphia, Pa., were used in the apparatus described. The nominal input resistance of the amplifier in recorder No. 1 is 7000 ohms and in recorder No. 2 is 400 ohms. The sensitivities of the two recorders are: No. 1, 50 mV full scale; No. 2, 1 mV full scale.

[***] The impedance convertor used in the present system is a DC amplifier developed by LOVELOCK[2].

[†] Switch S22 should have one position which is an open circuit (not shown in Fig. 1). This allows recorder No. 2 to be removed from the system if it is desired that recorder No. 1 only be in operation.

[††] A variable e.m.f. is not required when a recorder having a zero potential above the lower end of the scale is used.

This switching is necessary, otherwise a signal from the recorder which has gone off scale will cause a false answer to be plotted by the second recorder which is still on scale.

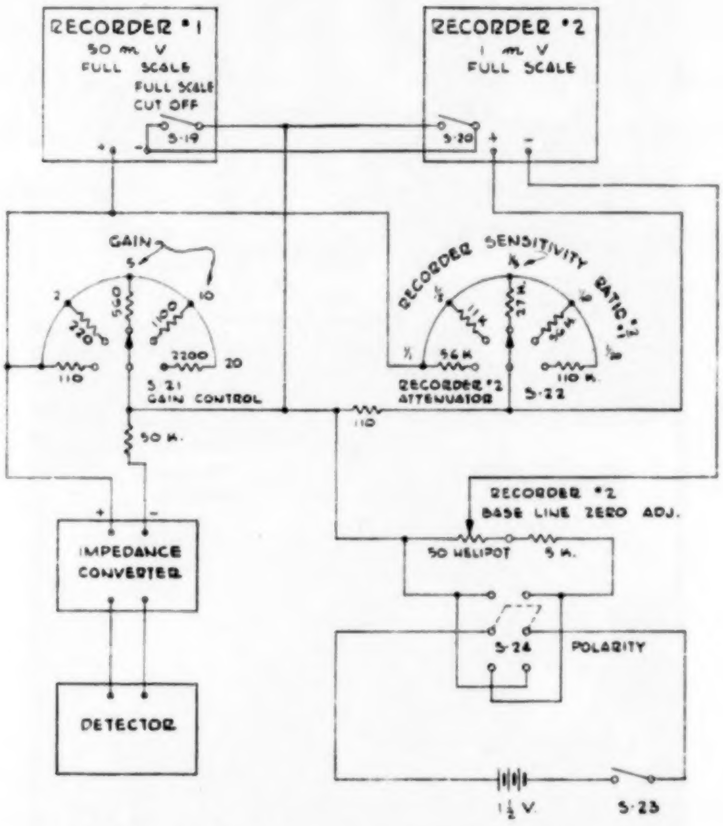
RECORDER #1
50 mV
FULL SCALE
FULL SCALE
CUT OFF
S-19
RECORDER #2
1 mV
FULL SCALE
S-20
+
−
GAIN
2
5
10
20
220
560
1100
2200
110
S-21
GAIN CONTROL
50 K.
RECORDER SENSITIVITY RATIO #2/#1
1/1
1/2
1/5
1/10
1/20
11 K
27 K
56 K
110 K.
RECORDER #2
ATTENUATOR
S-22
110
RECORDER #2
BASE LINE ZERO ADJ.
50 HELIPOT
5 K.
IMPEDANCE
CONVERTER
DETECTOR
S-24
POLARITY
1½ V.
S-23

Fig. 1. Schematic diagram for two-recorder operation.

The physical arrangement for this switching is shown in Fig. 2. The upper micro-switch seen in the lower recorder (No. 1) is switch S19. This switch is opened when the recorder reaches full scale by means of a cam which is an integral part of the recorder. In the upper recorder (No. 2) an identical cam is used to actuate switch S20. The actuating position of this cam must be changed when a change in recorder No. 2 attenuation is made. This is accomplished simply by setting recorder No. 1 to its full scale cut-off position and rotating the cam on the second recorder until it actuates switch S20.

Integration is accomplished by two ball and disc integrators attached to the pen action of the recorders as shown in Fig. 2. The integrators are located in the lower left corner of each recorder and are connected to the pen action of the recorder by means of an arm which rides on a special cam. Both of these items are supplied by the manufacturer of the integrator*. The integrators are adjusted so that a zero pulse rate output corresponds to a recorder pen position of zero.

The control panel and circuit diagram for the integrator-readout system are shown in Figs. 3 and 4. Energy for the signal formed by the integrators is supplied from dry cell batteries. Batteries are used rather than a line-operated supply to prevent integrator signal pickup by the impedance convertor-recorder system**. The signals

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* Model 25-1 Linear Integrators, manufactured by Librascope, Inc., Glendale, Calif., were used in the apparatus described.

** AC operation was not possible even when voltage regulation or an isolation transformer was used prior to the DC power supply. A linear response of the registers between zero and full scale pen deflection occurred only when the voltage supplied to the integrators was above 90 V.

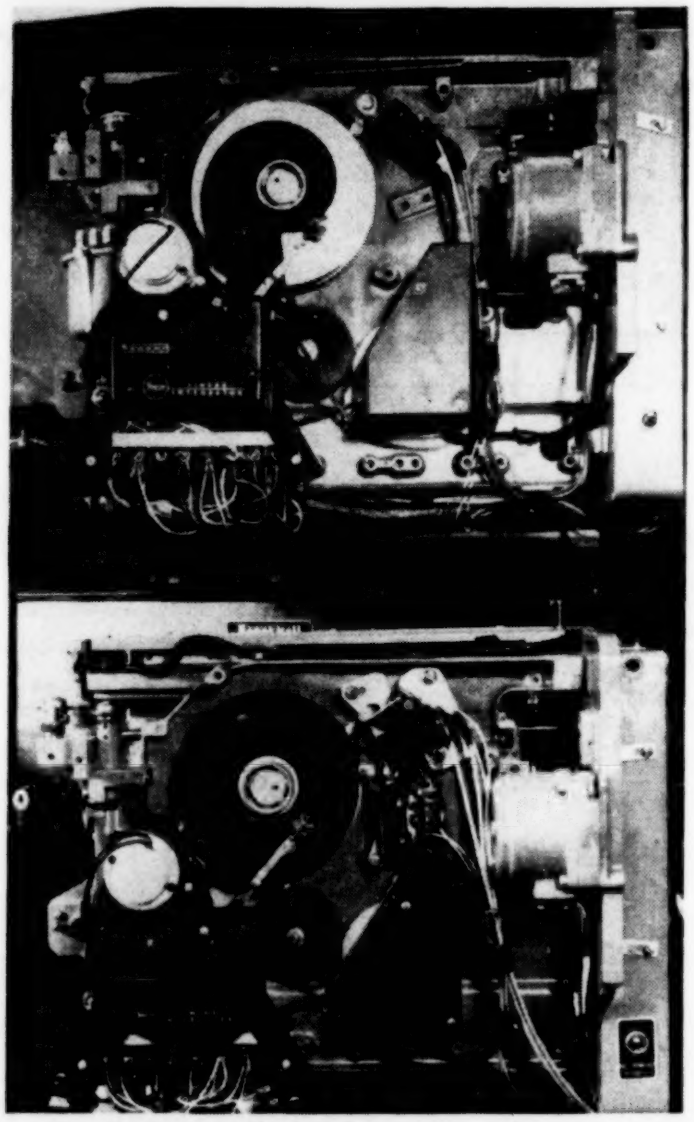

Fig. 2. Recorder switching arrangement for automatic advance and two-recorder operation. In the lower recorder is seen the arrangement of a single cam which operates three microswitches which control the automatic advance when the pen rises above the base line (S14, Fig. 4), disconnect the signal to the second recorder (S19) and disconnect the integrator signal to the registers (S18, Fig. 4) when the pen goes off scale. Switch S14 is the lower microswitch. Switches S18 (Fig. 4) and S19 are mounted parallel in the upper portion of the recorder. Switch S18 cannot be seen because it is directly behind switch S19. In the upper recorder is seen an adjustable cam which operates switch S20 which reactivates the lower recorder.

from the integrators operate high speed relays which in turn operate electric registers* capable of recording pulses at a maximum rate of at least 3000 per minute.

The signals from the integrators are shifted from one register to the next by means of rotary stepping relays** which are operated manually by button S2 or automatically by a microswitch on Recorder No. 1 (S14). Microswitch S14 is positioned so that it is actuated at the recorder pen position of zero, being in a closed position when the recorder pen is above the zero line. The physical arrangement of this switch is shown in Fig. 2. Switch S14 is the lower microswitch attached to the lower recorder. Adjustment of this switch to the zero position is accomplished simply by placing the pen in the zero position and rotating the cam until the switch is actuated. The magnitude of the deviation above the base line required to cause a shift in registers is adjustable by means of lowering the base line signal. This allows compensation, if necessary, for various degrees of noise in the base line signal. The minimum deviation of the recorder pen which will shift registers is about one-thousandth of full scale. A relay prevents any signal from reaching the registers until the pen is actually recording a signal above the base line. This relay is also operated by the base line microswitch, S14. In effect, as each peak is completed the next register is brought into operating position, but starts counting only when the next peak begins. If the apparatus is operated manually, this base line cancel should be eliminated by means of switch S16.

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* Form B159106, Series 15-91 registers, manufactured by Veeder-Root Inc., Hartford, Conn., were used.

** The stepping relays, Model SW-56-CDAC, are manufactured by the Automatic Electric Sales Corp., Chicago, Ill. The 48 V DC coils were replaced with 110 V DC coils.

The answers can be cleared automatically, when desired, by means of switch S6. This requires a built-in adjustable delay mechanism (R1) to allow for electrical reset of the registers. The delay is necessary because the rotary stepping relays normally pass through the reset position too quickly to operate the reset mechanism properly. Thus, each time a rotary stepping relay advances to the next peak position it moves through two switch positions. When the rotary switch reaches the first position, it is delayed, reset occurs, and then it moves rapidly to the final position. When the instrument advances to the next register it clears the register immediately ahead of the one that is counting. Although the register reset occurs only in the first of the two positions, counting occurs in both positions so that no loss of integrator signal takes place during the advance-reset cycle.

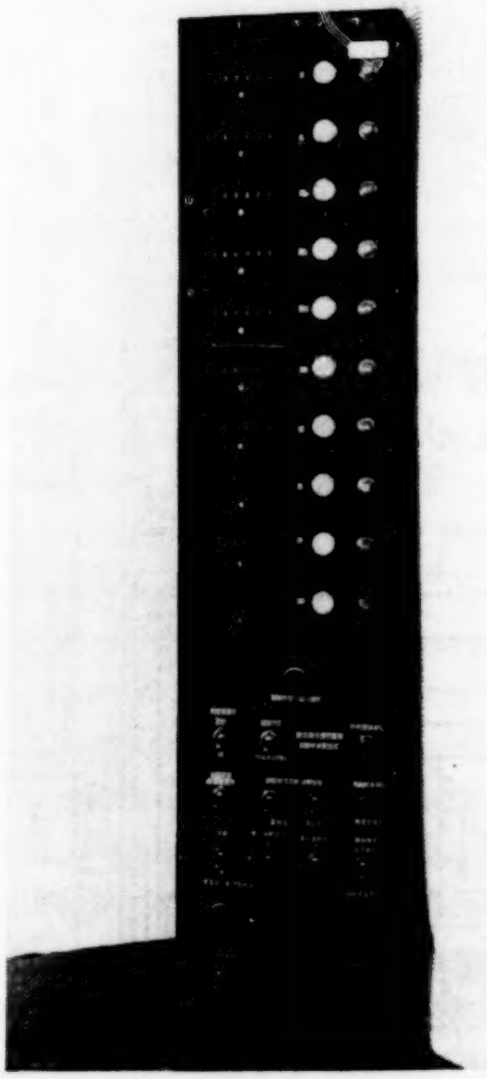

Fig. 3. Instrument panel of the automatic readout system.

Variable resistors (R2, R3) are included to allow the coils of the two rotary

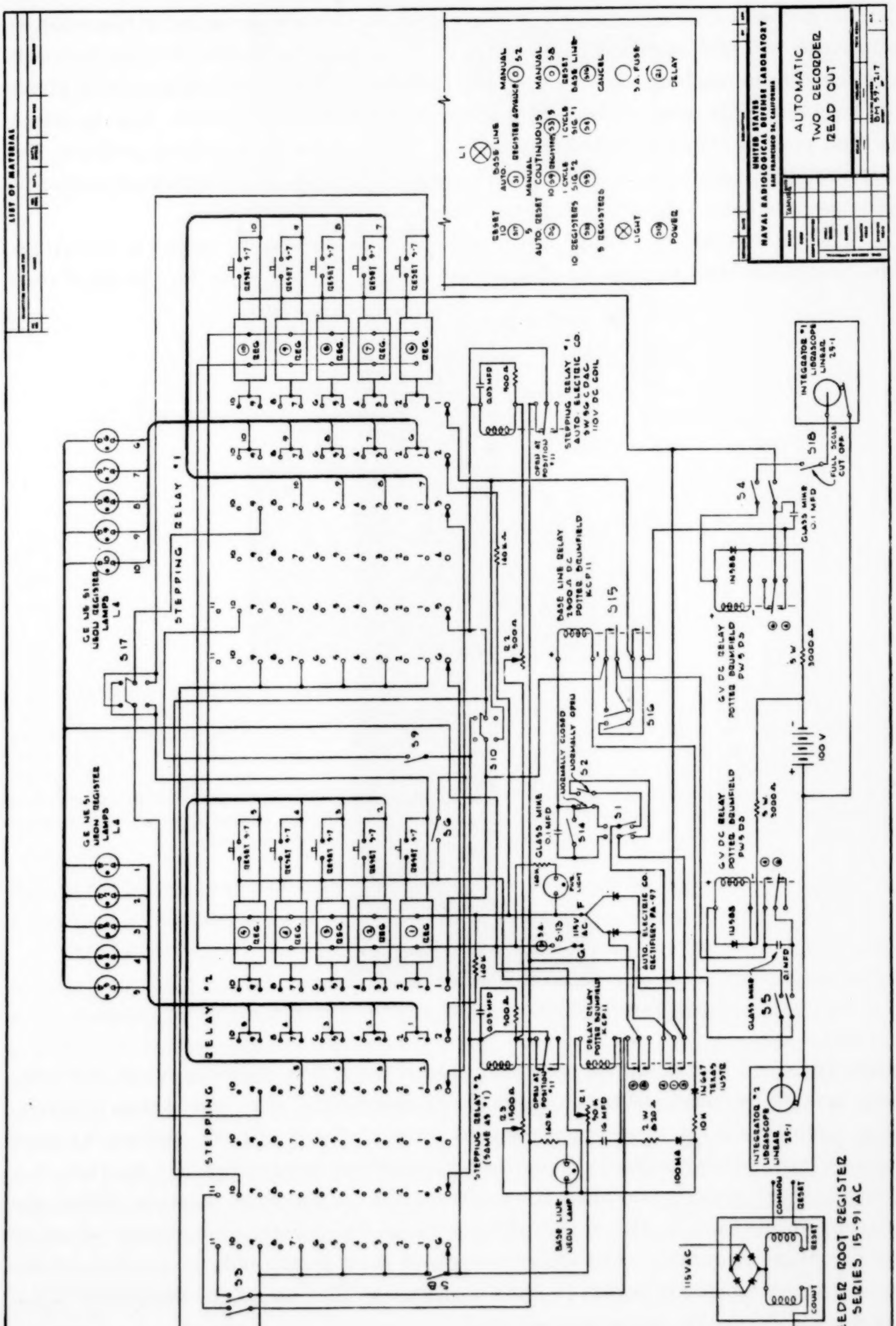
LIST OF MATERIAL
UNITED STATES NAVAL RADIOLOGICAL DEFENSE LABORATORY SAN FRANCISCO 24, CALIFORNIA
AUTOMATIC TWO RECORDER READ OUT
STEPPING RELAY #1
STEPPING RELAY #2
GE NE 51 NEON REGISTER LAMPS L4
STEPPING RELAY #1 AUTO. ELECTRIC CO. 110V DC COIL
STEPPING RELAY #2 (SAME AS #1)
BASE LINE RELAY POTTER BRUMFIELD KCP11
DELAY RELAY POTTER BRUMFIELD KCP11
6V DC RELAY POTTER BRUMFIELD PW5DD
AUTO. ELECTRIC CO. RECTIFIER PA-97
BASE LINE NEON LAMP
INTEGRATOR #1 LIBRASCOPE LINEAR 29-1
INTEGRATOR #2 LIBRASCOPE LINEAR 29-1
VEEDER ROOT REGISTER SERIES 15-91 AC
115VAC
COUNT
COMMON
RESET
100 V
115V AC
GLASS MIKE 0.1 MFD
FULL SCALE CUT OFF
RESET
AUTO.
MANUAL
BASE LINE
REGISTER ADVANCE
CONTINUOUS
10 REGISTERS
5 REGISTERS
SIG. #1
SIG. #2
CANCEL
LIGHT
POWER
DELAY
FUSE
S1
S2
S4
S5
S6
S8
S9
S10
S14
S15
S16
S17
S18

Fig. 4. Schematic diagram of the integrator-readout system.

stepping relays to be balanced in order to prevent the two registering systems (when two recorders are used) from getting out of phase. Resistor R2 is placed in front of the more sensitive of the two stepping relay coils. In order to balance the two coils resistor R3 is adjusted so that the weakest coil just responds when energized. Resistor R2 is then adjusted so that the more sensitive coil also just responds at the same setting of R3. After balancing is completed resistance R3 is removed from the circuit by adjustment of this resistor to its lowest resistance position.

Semiconductors and capacitors have been used to reduce arcing so that the instrument may be used near, and in conjunction with, high impedance circuits and so that the integrator commutators and relay points will give long service. In addition, semiconductors are used to prevent register counting when the system advances from one register to the next.

Operation of the various components of the instrument panel is outlined below. Reference should be made to Figs. 3 and 4. The light beside each register (L4) indicates which register is in position to receive a signal from the integrator system. A switch (S7) is also located beside each register. These switches (S7) are used for manual reset of individual registers.

The components at the bottom of the instrument panel are more clearly seen in Fig. 4. Switch S13 turns on the 110 V AC power to the register unit and "power-on" is indicated by the light immediately above switch S13. Switch S10 allows a choice to be made between the use of ten registers with one integrator or five registers with each of two integrators. Choice of which integrator is used is determined by switches S4 and S5. When switch S10 is placed in the five registers position, one or both signal switches (S4 and S5) can be actuated. When the latter switches are open, no signal reaches the registers from the integrators.

Automatic reset of the registers (as described previously) occurs when switch S6 is turned on. In order to clear the registers in the proper sequence, switch S17 must be set to the proper position depending on whether a single sequence of ten registers is used or two sequences of five registers are used. Switches S9 and S3 are used with one sequence of ten registers and two sequences of five registers, respectively. When these switches are placed in "continuous" position, cycling of the registers occurs, *i.e.*, after the last register in the sequence is used, the first register is brought back to operating position. When these switches are turned to the "one cycle" position, the answers are held on the registers and no further answers are recorded after the last register in the sequence is used.

Switch S1 determines whether advance from one register to the next is automatic or manual (as described previously). During automatic operation a light L1 indicates when the pen of recorder No. 1 is above the base line. Actuating push button switch S2 causes the registers to advance only once. Actuating switch S8 causes the registers to advance in sequence as long as the switch is depressed. This switch can be used to clear the registers (when S6 is on) and to remove all the registers from operating position (when S9 and S3 are in the "one cycle" position). The latter operation must be performed prior to changing from a ten register sequence to two five register sequences. Bringing the first into operating position for either the ten register sequence or two five register sequences is accomplished by momentary movement of switch S3 to the "continuous" position.

The base line cancel switch S16 shorts out the switch contacts of the base line relay. As previously described, during automatic operation this relay prevents any signal from reaching the register except when the pen of recorder No. 1 is above the base line. During manual operation this relay is not energized and, therefore, switch S16 must be on in order for the integrator signals to reach the registers. Switch S16 should be turned on when "zeroing in" the integrators because under this condition the integrator signal can be observed both above and below the base line.

Delay resistor R1 controls the time delay during automatic reset of the registers to zero. The resistance should be adjusted so that the delay is long enough to allow proper reset of the registers to occur.

PERFORMANCE

The system which has been described is fully automatic. Periodic base line adjustment of the detector signal, however, may become necessary depending upon the magnitude of the base line drift and the acceptable error. The rate at which peaks can be integrated and the answers stored is limited only by the speed with which the recorders can follow the detector signal.

An estimate of the precision of the system may be obtained by inspection of Figs. 5, 6 and 7. In Fig. 5 is shown the response of the two integrator-register systems

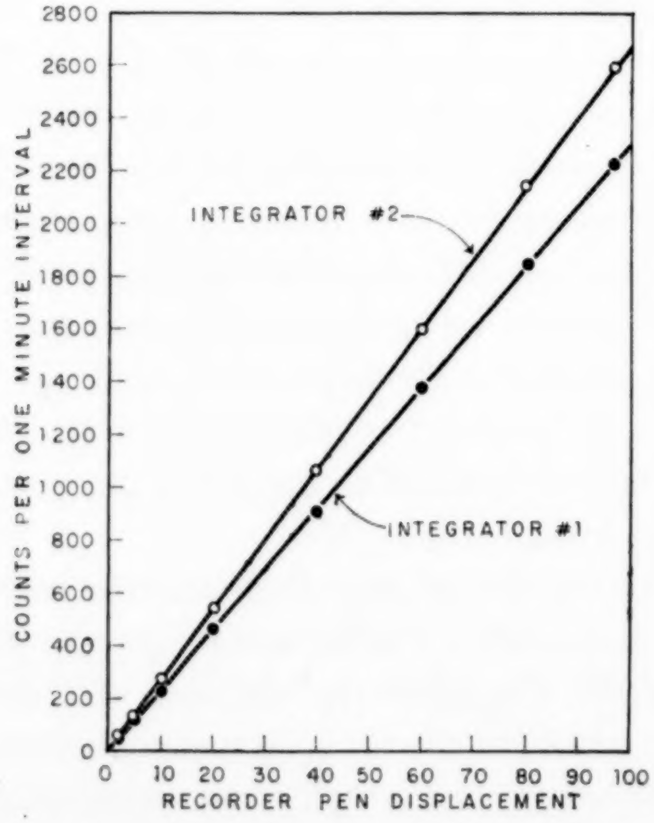

Fig. 5. Linearity of response of the integrator-register system to displacement of the recorder pens. Linearity of each integrator was checked separately. This was accomplished by adjusting the pen of the recorder to which the integrator was attached to various positions above the base line and measuring the register response for one minute.

to displacement of the recorder pens. It may be seen that the integrator signal outputs are linear even for small displacements of the recorder pen.

In Fig. 6 is shown the linearity of response of the integrator-register system to various amounts of methyl palmitate introduced into the gas chromatograph. The methyl palmitate was dissolved in isooctane and various volumes of this solution were used to obtain a standard curve. The data show that the numerical value obtained by the integration system is proportional to the amount of the methyl palmitate introduced, a property dependent on both a linear detector response and a linear integrator-readout response.

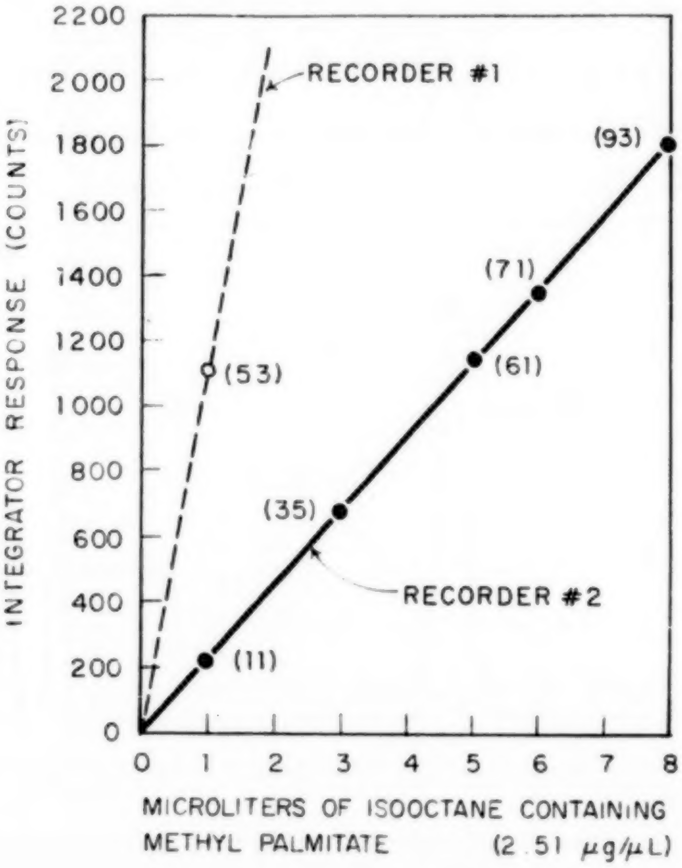

Fig. 6. Response of the integrator-register system to various amounts of methyl palmitate introduced into the chromatograph. Separation was carried out at 200° on an eight foot column packed with 30% Reoplex 400 (ref. [3]) and 70% 80–100 mesh Chromosorb W. Peak heights are indicated by values in parentheses (100 equals full scale). The retention time of methyl palmitate was 13 minutes.

A comparison of the areas computed by automatic integration and by planimetry is shown in Fig. 7. For the comparison, a sample of methyl esters of fatty acids obtained from rat liver mitochondrial phospholipids was used. Separation was carried out on an eight foot column containing Reoplex 400 as the liquid phase[3]. Direct planimetry of the recorder chart peak areas lying above the base line was performed with a Filotecnica planimeter of 0.02 square inch per unit of the vernier sensitivity. Reproducibility of areas determined by planimetry was within ± 3% of the mean for areas of more than 60 planimeter units. Integration with the apparatus described was performed automatically except for two manual register shifts indicated by the dotted lines. Some base line adjustment was necessary due to tailing of the solvent peak. The results indicate that the precision obtainable by the system described is good as judged by the constancy of the ratio of areas determined by the two methods.

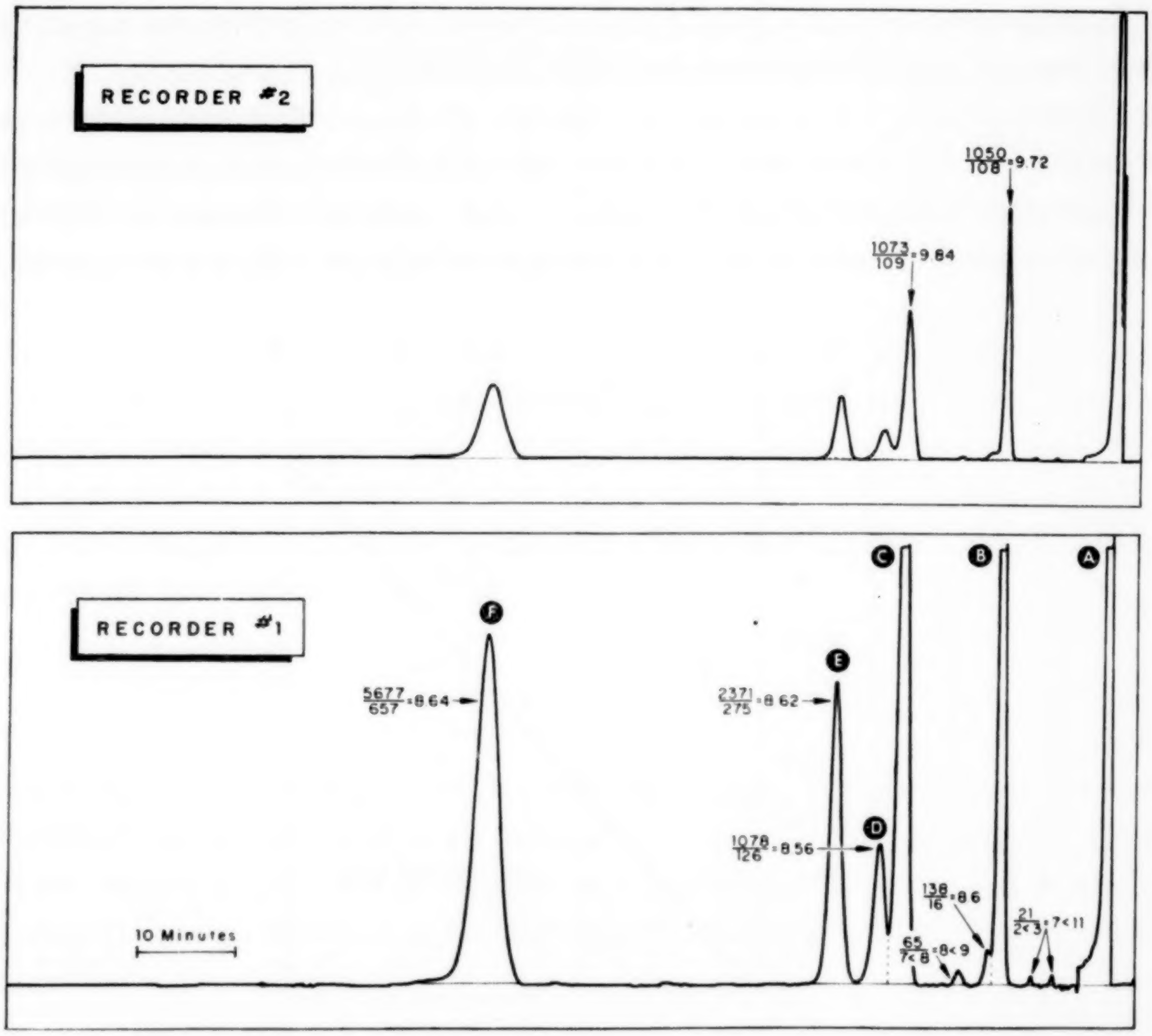

Fig. 7. A comparison of peak areas determined by the automatic integration-readout system and by direct planimetry of the recorder chart peaks. Chromatographic column data are the same as in Fig. 6. Recorder No. 2 was attenuated to one fifth the response of Recorder No. 1. Integration was performed automatically except where indicated by the dotted lines, at which time register advance was accomplished manually. Numbers above peaks indicate areas obtained by the two methods of area measurement. The numerator is the value obtained by automatic integration and the denominator is the value obtained by planimetry. The nearly constant ratio indicates good agreement between planimetry and automatic integration for both large and small peaks. The ratios obtained from Recorder No. 1 are not the same as those obtained from Recorder No. 2, because the sensitivities of the two integrators differ. Peak identification: (A) solvent; (B) palmitate; (C) stearate; (D) oleate; (E) lineoleate; (F) arachidonate.

SUMMARY

Details of construction and method of operation of an automatic two-recorder integration-readout system for gas–liquid chromatography have been presented.

The apparatus utilizes two recorders to plot data received from the detector. One recorder plots a fraction of the signal seen by the other recorder which, in effect, approximates an increase in recorder chart width. This allows a greater size range of sample components to be handled in a single chromatographic determination than is possible when only one recorder is used.

The area under each peak on each recorder is determined by integration as the data are plotted by the recorders. The pulsed signals obtained from the integrators

are stored on one of five registers available to each recorder. Advance of the signal from one register to the next and clearing of registers is automatically performed as each peak is completed.

Data are presented which indicate the precision of the apparatus.

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DETERMINATION OF INDIVIDUAL 17-KETOSTEROIDS BY GRADIENT ELUTION CHROMATOGRAPHY

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DINGEMANSE *et al.*[1] were first to describe a method of quantitative analysis for the major individual urinary 17-ketosteroids in micro amounts by use of alumina as an adsorbent and benzene, containing increasing concentrations of ethanol, as eluent. Subsequently, a number of adsorption methods were described in which various adsorbents and eluents were employed[2-6], but, in general, the overlapping of various fractions limited the efficiency of these procedures. This difficulty was overcome to some extent by the use of partition columns by JONES AND STITCH[7], who used nitromethane on silicic acid columns as the stationary phase, and later by EDWARDS AND KELLIE[8], who employed aqueous sodium acetate solution on silicic acid for the separation of the glucuronides of urinary steroid ketones. WILSON *et al.*[9] achieved the separation of urinary corticosteroids and C_{19} steroids on a micro column of aluminum silicate with 50% ethanol as the stationary phase by use of a "stepwise gradient" of hexane containing increasing concentrations of chloroform.

A major improvement in the analysis of individual 17-ketosteroids was accomplished by LAKSHMANAN AND LIEBERMAN[10, 11], through the use of gradient elution, which eliminates much of the "tailing" inherent in adsorption methods. KELLIE AND WADE[12] have recently described a very useful application of the gradient elution principle to the DINGEMANSE[1] method, in which a relatively simple device was used for producing the desired gradient automatically.

The development of a method in this laboratory for the determination of individual adrenocorticosteroids on water-impregnated silicic acid[13, 14] prompted us to investigate the possibilities of adapting this method to the fractionation of 17-ketosteroids. The gradient elution method described has the advantages of employing simple apparatus and lends itself well to completely automatic operation.

METHODS

1. *Column chromatography*

Just prior to packing a column, 7.5 ml of water, or 7.5 ml of water and 1.5 ml of methanol is added dropwise to 60 g of silicic acid while grinding it in a mortar. Since the water content of commercial silicic acid (Merck) as determined by heating several 1-g samples for 3 h and reweighing immediately, was found to be 15.8 ± 1%, this gives a water content of 25 %. This mixture is then suspended in water-saturated petroleum ether. The column is packed, and fractions of 400 drops each are collected, as described in the adrenal steroid method[13,14]. Gradient elution by petroleum ether, containing increasing amounts of methylene chloride, is effected by delivering the water-saturated solvents from two separatory funnels, mounted one on top of the other.

2. *Quantitative estimation*

The individual fractions, collected in test tubes, are allowed to dry and then 10 ml of 95 % ethanol is added to each tube. A 5-ml aliquot is withdrawn and set aside for subsequent identification by paper chromatography. The remaining aliquot is dried under vacuum at 40° in an automatic test tube evaporator. The dried residues in each tube are then analyzed by a modification of the M.R.C.[15] method of 17-ketosteroid determination. The modification involves the use of ascorbic acid-protected ethanolic 2.5 N KOH and development of the color at 0° for 3 h[16]. Calibration curves are determined for each compound[*] since they show an appreciable difference in response to the Zimmermann test.

3. *Paper chromatography*

Identification of the fractions is based on paper chromatography with the solvent systems methylcyclohexane/propylene glycol[17], ligroin/propylene glycol[18], and benzene–cyclohexane (1:1)/propylene glycol[19].

RESULTS AND DISCUSSIONS

Fig. 1 illustrates the fractionation obtained with the silicic acid column of 25 % water content. It was found that the separation is equally good with the commercial silicic acid to which the necessary amount of water is added and with the addition

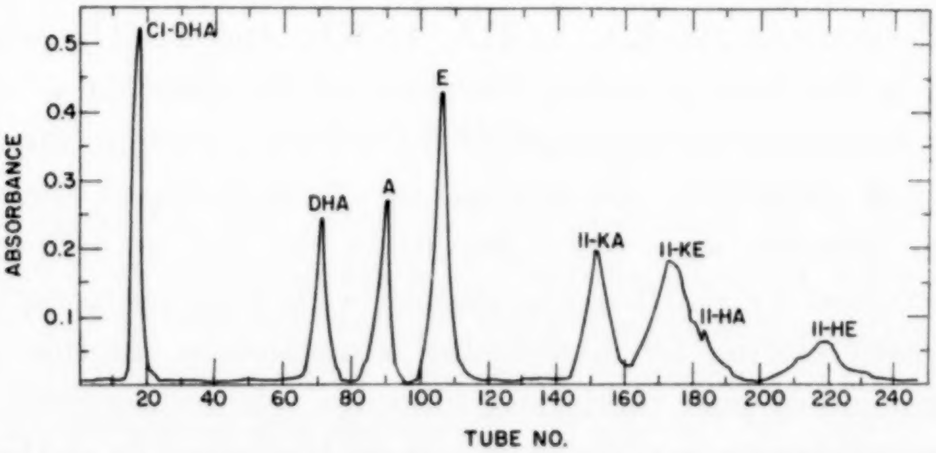

Fig. 1. Fractionation of 17-ketosteroids (100 γ of each) on a silicic acid column containing 25% water. Absorbance at 530 mμ after Zimmermann reaction.

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[*] Standard compounds used: 3α-hydroxyandrostan-17-one (androsterone, A); 3α-hydroxyandrostane-11,17-dione (11-ketoandrosterone, 11-KA); 3α,11β-dihydroxyandrostan-17-one (11-hydroxyandrosterone, 11-HA); 3α-hydroxyetiocholan-17-one (etiocholanolone, E); 3α-hydroxyetiocholane-11,17-dione (11-ketoetiocholanolone, 11-KE); 3α,11β-dihydroxyetiocholan-17-one (11-hydroxyetiocholanolone, 11-HE); 3β-chloro-Δ^5-androsten-17-one (chlorodehydroepiandrosterone, Cl-DHA); 3β-hydroxy-Δ^5-androsten-17-one (dehydroepiandrosterone, DHA).

of water to silic acid dried as described. All 17-ketosteroids used were completely separated, except 11-KE and 11-HA. Separation of these two substances is achieved by the use of 7.5 ml of water and 1.5 ml of absolute methanol per 60 g of commercial silicic acid (Fig. 2). This shifts the position of the elution peaks and somewhat decreases their sharpness, but there is no change in resolution. Repeated chromatograms have shown only minor variations in the position of elution peaks.

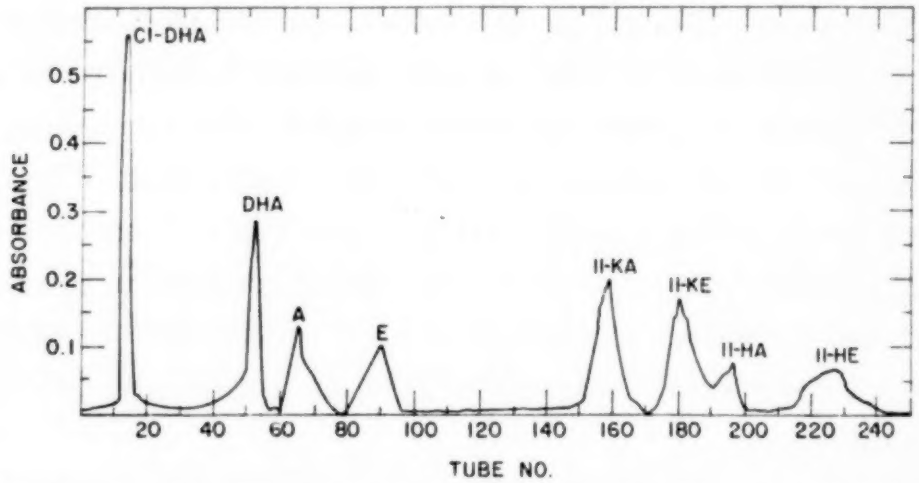

Fig. 2. Fractionation of 17-ketosteroids (100 γ of each) on columns of 60 g silicic acid plus 7.5 ml of water and 1.5 ml of methanol. Absorbance at 530 mμ after Zimmermann reaction.

Recoveries of each of the standard compounds, calculated with reference to individual calibration curves, are 95–100%, but lower for the mixed zone of 11-KE and 11-HA.

A number of experimental conditions were varied in order to test their effect on separation. In these experiments Cl-DHA and DHA were not included.

1. *Stationary phase of methanol–water (2:1)*

In preliminary experiments with 45 ml of this stationary phase per 60 g of dried silicic acid, partial separation of androsterone, etiocholanolone and their respective 11-oxygenated derivatives (11-KA, 11-HA, 11-KE, and 11-HE) was obtained, but elution occurred in the first 40 tubes. Decrease of the quantity of stationary phase to 40 ml greatly decreased the flow rate of the solvent. With 30 ml of the stationary phase, flow stopped altogether. An increase to 55 ml increased the resolution somewhat, but all the steroids were still collected in the first 40 tubes. An attempt to improve the resolution by starting the elution with pure petroleum ether and continuing the gradient elution by introducing a methylene chloride–petroleum ether mixture (7:3), instead of pure methylene chloride, was unsuccessful. Changes in the ratio of methanol:water to 4:1 and 1:1 caused the zones to coalesce and vitiated the partial resolution achieved by the 2:1 ratio.

2. *Propyl alcohol–water (2:1) and tert.-butanol–water (2:1)*

Changes in the polarity of the stationary phase by substitution of *n*-propanol and *tert.*-butanol for methanol resulted in each case in the elution of all six compounds in two peaks in the first ten tubes.

3. *Stationary phase of water*

In this series of experiments varying amounts of water were added to 60 g of the commercial silicic acid of known water content. Addition of 21 ml of water accomplished the separation of A and E in the first 40 tubes, but the remaining 4 steroids were eluted in a broad band in tubes 41 through 70. An increase in the water content to 36 ml shifted all six peaks to the first 40 tubes and gave poor resolution. Addition of 7 to 11 ml of water gave the same separation as shown in Fig. 1. However, when 6 ml of water or less was added, no resolution was obtained and the compounds were eluted in a broad zone extending over all 200 tubes analyzed. The effect of adding 1.5 ml of methanol to 7.5 ml of water has already been mentioned (Fig. 2). The quantity of methanol appears to be quite critical, and an increase to 2 ml broadens the chromatographic bands. The results obtained by use of 1.5 ml of methanol are readily reproducible.

It is apparent from the experimental results described that the relative participation of adsorption and partition in the fractionation on silicic acid columns varies with the water content of the stationary phase. This is in agreement with earlier studies of the effect of water on the adsorptive power of silicic acid[20,21]. KAY AND TRUEBLOOD[22] also indicate that water molecules may interfere with adsorption by occupying the adsorption sites on the column. They suggest that water can act as an adsorbent itself through hydrogen bonds and that methanol may be less effective in this respect because it is less apt to form hydrogen bonds. This effect may have some bearing on the improved separation of 11-KE and 11-HA obtained when a small amount of methanol is added to the water.

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SUMMARY

A method is described for the individual determination of eight 17-ketosteroids commonly found in urine. Fractionation of the compounds is achieved by use of a water-impregnated silicic acid column and elution with a concentration gradient of methylene chloride in petroleum ether. The effects of changes in the stationary phase on fractionation are presented and discussed.

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THE APPLICATION OF GAS CHROMATOGRAPHY TO THE DETERMINATION OF RETENTION DATA AND ACTIVITY COEFFICIENTS OF SOME ALKANES, ALKYL NITRATES, NITROALKANES AND ALCOHOLS ON SELECTED STATIONARY PHASES

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It has been realised for some time that gas chromatography can be applied to the determination of some physical constants of some compounds, and its use in the analytical field is ever widening. Activity coefficients at infinite dilution for a number of alkanes and other substances on polar and non-polar solvents have been calculated by KWANTES AND RIJNDERS[1], and for a number of halogenated hydrocarbons in dinonyl phthalate (DNP) and Silicone oil 702[2] from the data of POLLARD AND HARDY[3].

Little work on the gas chromatography of the simple alkyl nitrates has been reported[4,5]. GRAY AND PRATT[6] have calculated the latent heats of vaporisation of methyl, ethyl, *n*- and iso-propyl, *n*- and iso-butyl nitrates by normal physical methods.

The activity coefficients from gas chromatographic measurements are here determined for the series ethyl to *n*-octyl nitrate, nitromethane to 1-nitrobutane and *n*-pentane to *n*-nonane on squalane and DNP as solvents. The selectivity of diglycerol was such that reproduceable results were only obtained for the series of nitroalkanes and a series of normal alcohols.

The expression used here for the activity coefficients at infinite dilution is that derived by PORTER, DEAL AND STROSS[7] and KWANTES AND RIJNDERS[1], who have outlined the assumptions underlying its derivation. The expression is:

$$\gamma_p{}^0 = \frac{N_s RT}{k p_0}$$

where: N_s = the number of moles of solvent per unit volume at the column temperature T°K.

R = the gas constant.

p_0 = the vapour pressure of the pure solute at T°K.

k = the partition coefficient of the solute between the liquid and gas phases, given by:

$$V_R = V_m + kV_l$$

where: V_R = the retention volume, corrected for column pressure drop and temperature.

V_m = the dead volume of the column.

V_l = the volume of the solvent at T°K.

The retention volume, measured at room temperature, has to be corrected to column temperature, thus bringing about the replacement of column temperature by ambient temperature in the top of the expression.

EXPERIMENTAL

The apparatus closely followed SCOTT's design for routine analysis[8,9]. The column, of length 1 m, was heated by a vapour jacket containing successively boiling benzene, toluene and xylene, giving column temperatures of 80°, 111° and 139° respectively. The carrier gas was a 3:1 mixture of hydrogen and nitrogen whose flow, about 35 ml/min, was measured by a capillary flowmeter. The hydrogen flame detector was completely enclosed in the vapour jacket and so, to facilitate burning of the carrier gas at the jet, oxygen was introduced at about 110 ml/min. Inlet pressures were about 1.5 atmospheres.

The Pt–Ir/Rh–Au thermocouple, held 3 mm above the microflame, was maintained at column temperature; the thermo-e.m.f., balanced against a 4 V accumulator, was recorded on a 10 mV Elliot recorder.

The injection system was of SCOTT's design also, the capillary introducing samples of up to 1 μl. This is small enough to be within 1 % of the limiting case of zero sample size.

The solid support was 60–80 mesh ground firebrick, holding 20 % weight for weight of liquid phase. More than 15 % has been found necessary to ensure the independence of $\gamma_p{}^0$ from the amount of solvent present.

Samples of ethyl nitrate and *n*-butyl to *n*-nonyl nitrates were kindly supplied by the Shell Petroleum Co., Ltd., and 1-nitropropane by the Ministry of Supply Research Establishment, Waltham Abbey. 1-Nitrobutane was prepared from *n*-butyl bromide and silver nitrite, and after redistillation, gave only one peak on the chromatogram.

RESULTS

The specific retention volume, V_g, is defined as $V_R{}^0$, the retention volume fully corrected for dead volume, pressure drop across the column and measured at the column temperature, divided by the weight of the solvent.

Specific retention volumes and activity coefficients are shown in Tables I and II respectively, for the alkanes, alkyl nitrates and nitroalkanes on squalane and DNP. Specific retention volumes and activity coefficients of the alcohols and nitroalkanes on diglycerol are given in Table III.

TABLE I

SPECIFIC RETENTION VOLUMES ON SQUALANE AND DNP

Solvent	*Squalane*			*DNP*	
Temp.	*80°*	*111°*	*139°*	*80°*	*111°*
Pentane	24	—	—	15.5	—
Hexane	62.5	26.5	14	35.5	16.7
Heptane	145	54.5	27	80.5	34
Octane	330	115	49.5	147	71
Nonane	750	233	99	397	138
Ethyl nitrate	40	20.5	12	102	42
n-Propyl nitrate	105	42.5	22	215	80.5
n-Butyl nitrate	263	93	41.5	512	166
n-Amyl nitrate	610	194	88	1145	340
n-Hexyl nitrate	—	400	151	—	678
n-Heptyl nitrate	—	—	288	—	—
n-Octyl nitrate	—	—	550	—	—
Nitromethane	22.5	11.5	6.6	100	42.5
Nitroethane	48.5	23	13	193	71.5
1-Nitropropane	110	47	23.5	362	128
1-Nitrobutane	240	98	45.5	810	255

TABLE II

ACTIVITY COEFFICIENTS ON SQUALANE AND DNP

Solvent	*Squalane*			*DNP*	
Temp.	*80°*	*111°*	*139°*	*80°*	*111°*
Pentane	0.66	—	—	1.0	—
Hexane	0.65	0.70	0.66	1.1	1.1
Heptane	0.67	0.69	0.68	1.2	1.2
Octane	0.70	0.71	0.72	1.6	1.2
Nonane	0.77	0.78	0.80	1.4	1.3
Ethyl nitrate	1.9	1.7	1.6	0.74	0.73
n-Propyl nitrate	1.5	1.4	1.4	0.72	0.71
n-Butyl nitrate	1.3	1.2	1.2	0.68	0.68
n-Amyl nitrate	1.3	1.3	1.3	0.72	0.71
n-Hexyl nitrate	—	1.2	1.2	—	0.70
n-Heptyl nitrate	—	—	1.3	—	—
n-Octyl nitrate	—	—	1.3	—	—
Nitromethane	5.7	6.4	5.7	1.0	1.3
Nitroethane	3.7	4.2	3.2	0.90	1.2
1-Nitropropane	2.8	3.2	2.4	0.85	1.1
1-Nitrobutane	2.7	3.0	2.0	0.83	1.1

TABLE III

SPECIFIC RETENTION VOLUMES AND ACTIVITY COEFFICIENTS ON DIGLYCEROL

	80°		*111°*	
	V_g	γ_p^0	V_g	γ_p^0
Nitromethane	58.5	5.1	—	—
Nitroethane	48.5	9.2	—	—
1-Nitropropane	52	11	—	—
1-Nitrobutane	66	26	—	—
Methanol	74.5	1.1	26.5	1.1
Ethanol	67	2.0	24.5	1.7
n-Propanol	85	3.4	29	3.3
n-Butanol	122	5.4	38.5	4.9
n-Pentanol	176	6.5	51.5	6.4
n-Hexanol	263	12	66	11

DISCUSSION

Figs. 1 and 2 show graphically the relationships of log V_g and number of carbon atoms (Fig. 1) and log V_g and reciprocal of column temperature (Fig. 2).

As is to be expected from the theory of gas–liquid chromatography, there is a linear relationship between log V_g and number of carbon atoms (Fig. 1), from which other members of the same series might be identified. It is interesting to note the order of elution of an alkyl nitrate and the 1-nitroalkane of the same carbon number. For example, on squalane at 80°, ethyl nitrate is eluted before nitroethane, *n*-propyl nitrate before 1-nitropropane, but *n*-butyl nitrate *after* 1-nitrobutane. This is shown in Fig. 1 by the fact that the lines for these two classes of compounds cross between carbon numbers 3 and 4. Again, since the lines for the same two classes of compounds on DNP at 80° converge as the carbon number increases, one can predict that the two compounds of carbon number 7 will be eluted together.

The relative separation efficiencies of the two solvents squalane and DNP are indicated by considering the ratio of the specific retention volumes of two adjacent compounds of an homologous series. Squalane proves to be more efficient than DNP in this respect. For instance, the mean ratio of the specific retention volumes of adjacent straight-chain alkanes is 2.34 on squalane, but 2.27 on DNP at the same temperature, showing that squalane is a slightly better solvent. Corresponding figures for the nitroalkanes are 2.20 and 1.99 respectively, and for the alkyl nitrates 2.48 and 2.25 respectively.

However, for the separation of an alkyl nitrate form the nitroalkane with the same number of carbon atoms, DNP proves to be far more efficient than squalane. Defining the Separation Factor as the ratio of the specific retention volume of a nitroalkane and that of the alkyl nitrate of the same carbon number, we arrive at the following conclusions from results tabulated in Table IV.

From this table, DNP proves to be about 1.6 times more efficient for the three comparisons possible—namely for the ethane, propane and butane nitro- and nitrate

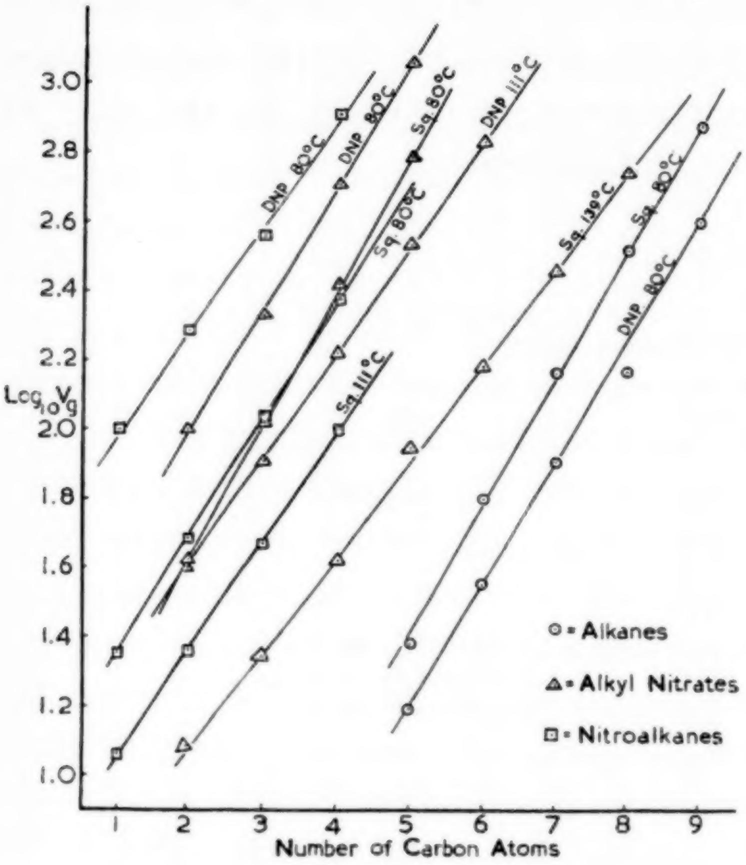

Fig. 1

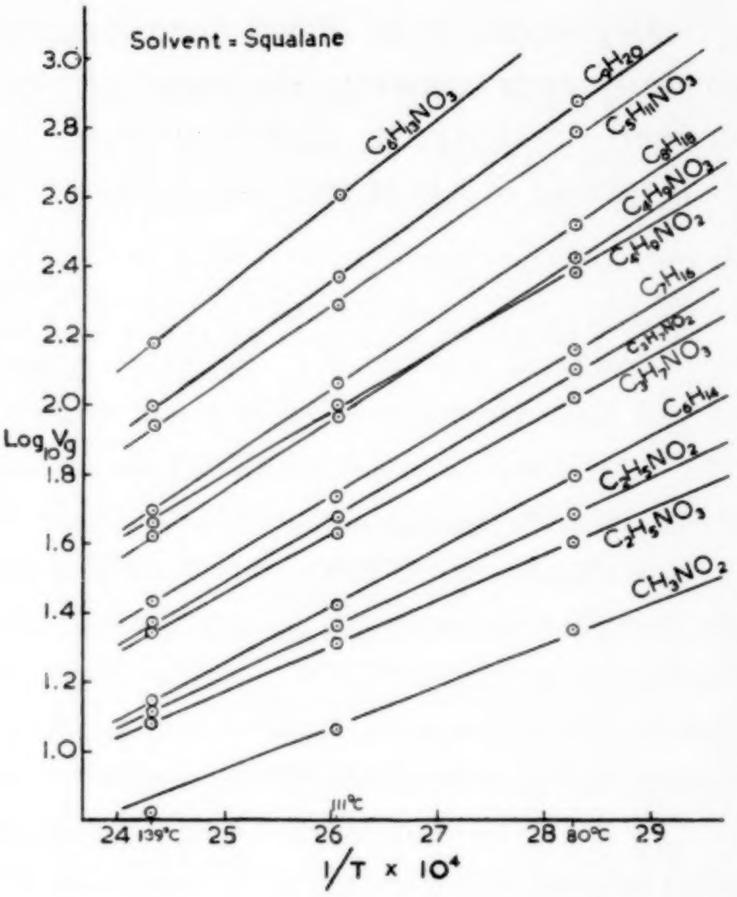

Fig. 2

derivatives. But it takes longer to effect these separations on DNP, since the V_g values are about 2.5 and 4.0 times higher for the alkyl nitrates and nitroalkanes respectively.

TABLE IV

SEPARATION FACTORS FOR NITROALKANES AND ALKYL NITRATES OF THE SAME CARBON NUMBER

Solvent	*Squalane*			*DNP*	
Temp.	*80°*	*111°*	*139°*	*80°*	*111°*
V_g (nitroethane) / V_g (ethyl nitrate)	1.21	1.12	1.08	1.89	1.70
V_g (1-nitropropane) / V_g (*n*-propyl nitrate)	1.05	1.10	1.07	1.68	1.59
V_g (1-nitrobutane) / V_g (*n*-butyl nitrate)	0.91	1.05	1.09	1.58	1.54

Heats of solution

From Fig. 2, heats of solution, ΔH_s, can be calculated by

$$\Delta H_s = 2.303 \times R \times \text{slope}.$$

Table V shows the values so obtained, compared with values of ΔH_v, the heat of vaporisation, for the alkyl nitrates and nitroalkanes.

ΔH_s varies from about 6–10 kcal/mole, and the value for an alkyl nitrate is generally approximately the same as that for the nitroalkane of the same carbon number—if anything, slightly higher. This situation is reversed in the case of ethyl nitrate (8.4 kcal/mole) and nitroethane (8.9 kcal/mole).

TABLE V

HEATS OF SOLUTION OF ALKYL NITRATES AND NITROALKANES ON SQUALANE AND DNP IN KILOCALORIES/MOLE

	Squalane	*DNP*	ΔH_v
Nitromethane	5.9	7.3	8.2(ref.[10])
Nitroethane	6.6	8.9	—
1-Nitropropane	7.5	9.0	—
1-Nitrobutane	8.1	9.9	—
Ethyl nitrate	5.9	7.8	7.92(ref.[6]) at b.p.
n-Propyl nitrate	7.6	8.4	8.58(ref.[6]) at b.p.
n-Butyl nitrate	9.1	9.9	9.40(ref.[6]) at 120°
n-Amyl nitrate	9.6	10.8	—

Vapour pressures

The vapour pressure of the pure solute appears in the expression for γ_p^0. Vapour pressure–temperature relationships for the alkanes and nitroalkanes were readily available[10]. For ethyl, *n*-propyl and *n*-butyl nitrates, the equations derived by GRAY AND PRATT[6] were used. These are of the form:

$$\log_{10} p(\text{mm Hg}) = A - \frac{B}{t(^\circ\text{C}) + D}$$

where A, B and D are constants for a given nitrate. These equations give good straight lines for $\log_{10} p$ against $1/T$ in the range 80–139°. The only information about vapour pressures of the higher nitrates was the following:

Compound	*Boiling point (°C)*
n-Butyl nitrate	34–36/15 mm Hg
n-Amyl nitrate	61/22 mm Hg
n-Hexyl nitrate	68/15 mm Hg
n-Heptyl nitrate	83–84/15 mm Hg
n-Octyl nitrate	100/13 mm Hg

The boiling points of *n*-amyl and *n*-hexyl nitrates were determined at 760 mm Hg as 157° and 180° respectively. *n*-Heptyl and *n*-octyl nitrates decomposed on heating. Thus two points were available for the *n*-amyl and *n*-hexyl nitrates and only one for the *n*-heptyl and *n*-octyl nitrates. From this information, and coupled with the more comprehensive information for the ethyl, *n*-propyl and *n*-butyl nitrates, the "most likely" lines were drawn for the *n*-heptyl and *n*-octyl nitrates, using the single point available for each nitrate.

Activity coefficients

Tables II and III show the values for γ_p^0 obtained on squalane, DNP and diglycerol. Those for the alkanes on squalane agree substantially with those obtained by Kwantes and Rijnders[1]. For example, Kwantes and Rijnders calculate the values of γ^0 for heptane at 80°, 105° and 135° to be 0.68, 0.66 and 0.68 respectively. Our values at 80°, 111° and 139° are 0.67, 0.69 and 0.68 respectively. These results ($\gamma_p^0 < 1$) indicate the system of a non-polar solute in a non-polar solvent. The results for the nitroalkanes on squalane are not very reproduceable, and this is probably due to the residual adsorption effects of the solid support on the polar solute. Kwantes and Rijnders found that for all systems of the polar solute on non-polar solvent, skew peaks were given, with tailing, and retention times were dependent on sample size on all types of inorganic support. We observed tailing peaks for the nitroalkanes on squalane, using ground firebrick as support, and γ_p^0 was > 1, indicating a marked difference in polarity of the solute and the solvent. So it seems clear that the nature of the solid support was influencing the retention time of the solute. This effect might have been nullified by the use of an inert support such as metal helices[1].

For the alkyl nitrates on squalane, γ_p^0 is only just > 1, and not far greater than the figure for the corresponding alkane of the same carbon number. This would indicate that the molecule $R \cdot O \cdot NO_2$ is considerably less polar than the corresponding nitroalkane $R \cdot NO_2$. Moreover, the values of γ_p^0 for the alkyl nitrates on squalane are quite consistent with temperature, indicating that the effect of the solid support was negligible.

SUMMARY

Retention data are obtained for some normal alkanes, nitroalkanes, alkyl nitrates and alcohols on the stationary phases squalane, dinonyl phthalate and diglycerol. The relative separation efficiencies of the two solvents squalane and dinonyl phthalate with respect to the nitrogen-containing series, and activity coefficients and heats of solution are calculated. The relative polarities of some of the solutes and solvents and the effects of the solid support are also discussed.

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SUPPLEMENTARY DATA FOR THE PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PHENOL DERIVATIVES AND RELATED COMPOUNDS OF BIOCHEMICAL INTEREST, USING A "REFERENCE SYSTEM"

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I. INTRODUCTION

The present investigation should be regarded as a supplement to a previous series of investigations on phenolic derivatives and related compounds published in this Journal[1]. In the earlier studies attention was mainly focused on substances found as metabolic products in mould cultures and on the possible application of this procedure to investigations within related fields.

A great number of closely related compounds was studied by paper chromatography in order to establish a systematic relationship between the structure and the R_F value, six different solvent systems and ten colour reagents being used in this investigation. The aim was to find a means of predicting, with a certain degree of probability, whether several functional groups occur in phenolic compounds when unknown mixtures or a desired type of compound have to be examined. It was found that there was a close connection between the structure and the different colour reactions, which followed certain rules, especially in the case of aromatic nuclei containing several hydroxy groups. Thus 1,2-, 1,3- and 1,4-dihydric phenol derivatives show significant characteristics when their colour reactions are compared with each other. From the R_F values of these three types of compounds in several solvent systems, it was possible at the same time to establish with certainty the presence of two hydroxy groups.

In order to extend to several types of compounds the investigations regarding the effect of various substituents on the colour and R_F value, some new data were collected and these are presented in this paper. These new data may be useful for the identification and description of aromatic compounds, not only those of vegetable origin, but also those occurring as metabolic products of living organisms, *e.g.* compounds originating from tryptophan, tyrosine and phenylalanine metabolism. Metabolic disorders have for many years been the object of intensive studies by several investigators and a great number of papers have appeared (for references see ARMSTRONG *et al.*[2], TOMPSETT[3], BOSCOTT AND BICKEL[4], and BOYLAND AND WILLIAMS[5]).

The various mechanisms of detoxification of aromatic compounds, some of which are manifested by the excretion of hippuric acids, also belong to this subject and have been investigated independently or in connection with drug metabolism. Recently, interest has been centred on compounds with an indole structure, since it has been claimed that they are of significant importance in certain mental diseases, either because they are responsible to some extent for the condition or because they occur in the patients in greater amounts than are found in normal persons[6–9]. Further investigations in this direction would therefore be worthwhile. For this purpose the previous collection of compounds has been supplemented by several new types of compounds closely connected with the above-mentioned problems, and these were also submitted to paper-chromatographic analysis in several solvent systems.

2. MATERIALS AND METHODS

For one-dimensional descending chromatography rectangular glass jars of size 20 × 30 × 60 cm were used.

Whatman No. 1 filter paper with the dimensions 24 × 48 cm was employed.

The composition of the six solvent systems is given in the section "Abbreviations used in the Tables".

For spraying, the following ten standard reagents were used throughout the experiments:

Diazotized sulphanilic acid (obtained from Th. Schuchardt Co., Munich); diazotized 4-benzoylamino-2,5-dimethoxyaniline (Light Chemicals Co., England); diazotized *o*-dianisidine (Light Co.); *p*-nitrobenzenediazonium fluoborate (Eastman Kodak, U.S.A.); 2,6-dibromoquinone-4-chloroimide; 2,4-dinitrophenylhydrazine; ferric chloride; phosphomolybdic acid; potassium permanganate; bromophenol blue. For the composition of these reagents, see the section "Abbreviations used in the Tables".

Additional reagents: For indole derivatives the Ehrlich reagent[10–12], 1% solution of *p*-dimethylaminobenzaldehyde (abbreviated as EH) in 1 *N* HCl, was used. In order to obtain more distinctive shades and more strongly coloured spots varying from yellow to pink, blue and green, another similar reagent was introduced, which proved to be very useful although the background colour became more dominant after some time, owing to the oxidation of the reagent in the air. This reagent was prepared by dissolving *p*-dimethylaminocinnamaldehyde (obtained from Heidenheimer Chemisches Laboratorium, Heidenheim-Brenz, Germany) in 1 *N* HCl so that a 0.1% solution (abbreviated as DAC) was obtained. In most cases the coloured spots developed instantaneously. The use of 2-chloro-4-dimethylaminobenzaldehyde (Light Co.) in hydrochloric acid had no marked advantage over the classical Ehrlich reagent; in many cases it gave no reaction at all with indole derivatives.

In general these reagents gave yellow, red, violet, blue, green and grey colours with indole derivatives. However, alkaloids with an indole skeleton, such as reserpine, yohimbine, physostigmine, brucine and strychnine failed to react under the prevailing conditions. Also some other types of compounds gave colours, *e.g.* urea (EH – yellow, DAC – carmine), thyroxine (EH – yellow, DAC – magenta), kynurenine (EH – orange, DAC – carmine), histidine (EH – yellow, DAC – nil), *p*-aminobenzoylglutamic acid (EH – orange, DAC – red violet), 2,5-diaminobenzoic acid (EH – carmine, DAC – blue), N-*p*-hydroxyphenylglycine (EH – yellow, DAC – magenta), N-*o*-carboxyphenylglycine (EH – orange, DAC – carmine), formylglycine (EH – yellow, DAC – orange), ureidosuccinic acid (EH – yellow, DAC – pink), histamine (EH – nil, DAC – orange), sarcosine (EH – nil, DAC – orange), hydantoin (EH – nil, DAC – carmine), hydantoic acid (EH – yellow, DAC – carmine), allantoin (EH – pale yellow, DAC – yellow) and allantoic acid (EH – pale yellow, DAC – carmine), and *m*- and *p*-nitrohippuric, 3,5-dinitrohippuric and *p*-aminohippuric acids (EH – yellow, DAC – carmine).

For detecting hippuric acid derivatives a 2% solution of recrystallized *p*-dimethylaminobenzaldehyde (abbreviated as DAB) in acetic anhydride was used[13,14]. This reagent gave yellow, orange and pink colours with hippuric acid derivatives; some of these derivatives, however, gave weak shades. On the other hand, closely related compounds such as formylglycine, α-aminophenylacetic acid, N-*o*-carboxyphenylglycine, *p*-aminobenzoylglutamic acid, creatinine, betaine and hydantoic acid gave yellow colours. Compounds such as phenoxyaceturic acid, phenaceturic acid, N-phenacetylglutamic acid gave no colour reaction.

Even compounds like *o*-hydroxyphenylacetic acid and homogentisic acid show yellow colours with DAB reagent after 24 hours.

As noticed earlier by OSTEUX AND LATURAZE[15], the hippuric acid reagent gave reddish colours with some aliphatic compounds such as *cis*- and *trans*-aconitic acid, aconitic anhydride. In order to establish the possible disturbances that might arise from other compounds than hippuric acid derivatives, the aliphatic compounds listed in Tables XVIII to XXI in the previous publication[1] and Table XV in the present paper, were tested. Weak yellow colours that could hardly be distinguished from the background colour at this concentration level were observed with the following compounds: malic acid, fumaric acid, itaconic acid, mesaconic acid, pyruvic acid, β-ketoglutaric acid, citric acid and mesoxalic acid. The spots were, however, more discernible in U.V. light.

In an attempt to obtain clearer differences in colour between the hippuric acid derivatives a 1% solution of *p*-dimethylaminocinnamaldehyde and a 2% solution of 2-chloro-4-dimethylaminobenzaldehyde in acetic anhydride were also tested. The former was shown to be fairly reactive, giving stronger red-brownish colours, but it was not so easy to distinguish the shades from each other. The reagent prepared from the chloro-derivative did not show any advantage over the above-mentioned reagents as a detecting agent for hippuric acids.

For detecting alkaloids, a Dragendorff reagent[16–18] was prepared by dissolving 2 g $KI \cdot BiI_3$ (obtained from Merck Co., Darmstadt) in 100 ml 0.01 *N* HCl and filtering the hot solution. This reagent gave orange colours with alkaloids, and it proved possible to detect the latter when the other reagents failed to react.

In general the paper-chromatographic procedure followed essentially the same principles as described earlier[1]. The colours obtained with the different reagents were recorded as numbers, following the "Derwent" colour pencils index of Cumberland Pencil Co., Keswick, England. The description of these colours is found under the heading "Colour Index for the Tables".

The compounds investigated were mainly obtained from commercially available preparations. The hydroxy-derivatives of phenyllactic and phenylpyruvic acids were synthetized by Dr. B. WENGLE in this laboratory, to whom the author is greatly indebted.

The various hippuric acid derivatives used in this investigation were synthetized by the author, mainly according to two methods and on a scale of *ca.* 10 mmole.

Synthesis of hippuric acid derivatives

(1) Substituted non-hydroxylated hippuric acid derivatives were in general synthetized according to the procedure described by NOVELLO *et al.*[19] for *o*-chlorohippuric acid, by shaking a glycine solution in sodium hydroxide with the corresponding benzoyl chloride and recrystallizing the product from a minimum amount of hot water.

Ortho-, *meta-* and *para*-hydroxy-hippuric acids were prepared according to FISCHER[20], the OH-group of the phenolic acid being first protected by treatment with chloroformic acid ethyl ester, which yields an O-carbethoxy derivative. The corresponding acid chloride was subsequently obtained by treating this derivative with phosphorus pentachloride. The substituted benzoyl chloride was then condensed in ether with an excess of glycine ethyl ester. The precipitated glycine ester hydrochloride was removed and the residue was hydrolysed with 1 *N* sodium hydroxide; the hippuric acid derivative was isolated after acidification and was finally recrystallized from water.

(2) The second type of synthesis was carried out according to SHEEHAN AND HESS[21], and ITO AND NEILANDS[22], whereby equimolar amounts of the corresponding hydroxy- or dihydroxy-benzoic acid and glycine ethyl ester (obtained by treating glycine ethyl ester hydrochloride with silver oxide in tetrahydrofuran and filtering off silver chloride) in tetrahydrofuran were condensed with dicyclohexyl-carbodiimide in slight excess, under nitrogen atmosphere, overnight at room temperature. Acetic acid was added to decompose the condensing agent. Urea was removed and the solvent was evaporated to dryness. The ester residue was hydrolysed with 1 *N* NaOH at room temperature for four hours, and the product was isolated, after acidification, by extraction with ethyl acetate. It was recrystallized in a minimum amount of hot water. The yields were about 50% in the case of *o*-hydroxyphenylacetylglycine.

This method has proved suitable and more convenient for hydroxylated derivatives, since the steps of protecting the hydroxy groups by converting them into O-carbethoxy groups is eliminated. Especially when synthetizing dihydroxy-derivatives the protection of both OH-groups by chloroformic acid ethyl ester is rather tedious, since usually only one group can be protected at a time (see FISCHER[23]).

α-Hydroxyhippuric acid was synthetized according to HAAS[24], whereby hippuric acid, suspended in carbon tetrachloride, was oxidized with bromine in the presence of red phosphorus. The product was recrystallized several times from water.

It is intended to publish more detailed reports on the syntheses of various hippuric acids, as well as data on the occurrence of hippuric acids in urine.

3. GUIDE TO TABLES I–XV

The following Tables present information compiled for approx. 270 organic compounds, investigated by the procedure outlined above. The R_F values are recorded in six different solvent systems designated by F, E, A, B, C and D. For the composition of these solvents, see the list of abbreviations given below. Under the heading "Detection" (columns 2–11) the colour reactions are recorded for 10 different reagents used for the identification of each compound; in addition, the colour produced in ultraviolet light is indicated in the first column under this heading. The amount of substance used in these experiments was 25–50 γ per spot, which produced different colours when the reagents were applied. These colours are referred to by numbers, the explanation of which is found in the colour index. For abbreviations and the composition of the reagents, the reader is referred to the following section.

Table I	Monohydric phenols and their derivatives.
Table II	1,2-Dihydric phenols and their derivatives.
Table III	1,3-Dihydric phenols and their derivatives.
Table IV	1,4-Dihydric phenols and their derivatives.
Table V	Trihydric phenols.
Table VI	Naphthalene and quinoline derivatives.
Table VII	Benzoic and phthalic acid derivatives.
Table VIII	Aromatic non-phenolic and heterocyclic monocarboxylic acids with the COOH group in the side chain, and their derivatives.
Table IX	Pyridinedicarboxylic acids, non-hydroxylated.
Table X	Aromatic, heterocyclic and some aliphatic amino acid derivatives.
Table XI	Biologically active nitrogen compounds, *e.g.* biogenic amines, etc.
Table XII	Indole derivatives.
Table XIII	Natural products of vegetable and mould origin, *e.g.* alkaloids, antibiotics, etc.
Table XIV	Hippuric acid derivatives.
Table XV	Miscellaneous aliphatic carboxylic acids.

ABBREVIATIONS USED IN TABLES I–XV

Chromatographic solvent systems:

F = Ethyl methyl ketone–acetone–formic acid–water (40:2:1:6 parts by volume)
E = Ethyl methyl ketone–diethylamine–water (921:2:77 parts by volume)
A = Methyl isobutyl ketone–formic acid–water (10 parts ketone saturated with 1 part 4% formic acid)
B = Chloroform–methanol–formic acid–water (10 parts of chloroform saturated with a mixture of 1 part methanol and 1 part 4% formic acid)
C = Benzene–ethyl methyl ketone–formic acid–water (a mixture of 9 parts benzene and 1 part ketone saturated with 1 part 2% formic acid)
D = Benzene–formic acid–water (10 parts benzene saturated with 1 part 2% formic acid)

Reagents used for detection:

U.V. = Ultraviolet light
D1 = Diazotized sulphanilic acid (0.3% solution in dioxane–water 1:2)
D2 = Diazotized 4-benzoylamino-2,5-dimethoxyaniline (0.6% solution in dioxane–water 1:2)
D3 = Diazotized *o*-dianisidine (0.6% solution in dioxane–water 1:2)
D4 = *p*-Nitrobenzenediazonium fluoborate (0.4% solution in dioxane–water 1:2)
DB = 2,6-Dibromoquinone-4-chloroimide (0.5% solution in dioxane–acetone 4:1)
DN = 2,4-Dinitrophenylhydrazine (*ca.* 0.1% solution in 1 *N* HCl)
Fe = Ferric chloride (2% aqueous solution)
Mo = Phosphomolybdic acid (2% aqueous solution)
Mn = Potassium permanganate (1% aqueous solution)
Ind = Bromophenol blue (*ca.* 0.05% solution in ethanol)

Special reagents used for detection:

NH = Ninhydrin reagent (2% solution in butanol saturated with water)
EH = Ehrlich reagent (1% *p*-dimethylaminobenzaldehyde in 1 *N* HCl)
DAC = *p*-Dimethylaminocinnamaldehyde (0.1% solution in 1 *N* HCl)
DAB = *p*-Dimethylaminobenzaldehyde (2% solution in acetic anhydride)
Bi = Dragendorff reagent (2% solution of potassium bismuth tetraiodide in 0.01 *N* HCl)

Owing to the lack of space in the tables, the R_F values have been recorded as 12, 56, 88, but should be read: 0.12, 0.56, 0.88 etc. The colours produced by the reagent are recorded as numbers, and the corresponding shades can be found in the colour index. The —sign means that no reaction was observed under the prevailing conditions. The +sign indicates an uncertain reaction, which was too weak to deserve colour estimation. Reactions with the reagents Mn and Ind are only indicated by the signs: —, + or ++. The ++sign means that a positive reaction was obtained immediately. In the few cases where the colours are recorded by a number placed on top of another number, *e.g.* $\left.\begin{matrix}24\\57\end{matrix}\right\}$, this indicates that immediately upon spraying a violet spot (24 in the colour index) appears which, within a few seconds, turns to light brown (57 in the colour index). Usually most colours are unstable and after some time take on a brownish tone, which is to some extent caused by the chemical influence of other reagents used in the vicinity. This change in colour is not recorded in the tables, nor is there any column for those compounds that, at this low concentration, are visible on the chromatograms because of their own colour.

The various compounds listed in the tables have been arranged as far as possible according to the following principles:

(1) Free phenols together with their methoxy- and alkyl-derivatives; (2) the corresponding amino- and nitro-derivatives; and (3) further derivatives in the order: hydroxymethyl, keto, aldehyde and finally monocarboxylic acid derivatives, derivatives with a carboxylic acid group in the side chain and dicarboxylic acids.

COLOUR INDEX FOR THE TABLES

The colours that were produced by the action of different reagents on the investigated compounds listed in Tables I–XV, have been recorded as numbers according to the following code:

01	Zinc Yellow	25	Dark Violet	49	Sap Green
02	Lemon Cadmium	26	Light Violet	50	Cedar Green
03	Gold	27	Blue Violet Lake	51	Olive Green
04	Primrose Yellow	28	Delft Blue	52	Bronze
05	Straw Yellow	29	Ultramarine	53	Sepia
06	Deep Cadmium	30	Smalt Blue	54	Burnt Umber
07	Naples Yellow	31	Cobalt Blue	55	Vandyke Brown
08	Middle Chrome	32	Spectrum Blue	56	Raw Umber
09	Deep Chrome	33	Light Blue	57	Brown Ochre
10	Orange Chrome	34	Sky Blue	58	Raw Sienna
11	Spectrum Orange	35	Prussian Blue	59	Golden Brown
12	Scarlet Lake	36	Indigo	60	Burnt Yellow Ochre
13	Pale Vermilion	37	Oriental Blue	61	Copper Beech
14	Deep Vermilion	38	Kingfisher Blue	62	Burnt Sienna
15	Geranium Lake	39	Turquoise Blue	63	Venetian Red
16	Flesh Pink	40	Turquoise Green	64	Terra Cotta
17	Pink Madder Lake	41	Jade Green	65	Burnt Carmine
18	Rose Pink	42	Juniper Green	66	Chocolate
19	Madder Carmine	43	Bottle Green	67	Ivory Black
20	Crimson Lake	44	Water Green	68	Blue Grey
21	Rose Madder Lake	45	Mineral Green	69	Gunmetal
22	Magenta	46	Emerald Green	70	French Grey
23	Imperial Purple	47	Grass Green	71	Silver Grey
24	Red Violet Lake	48	May Green	72	White=colourless

TABLE I

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME MONOHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
90	93	92	92	93	92	2,3,6-Trimethylphenol	—	09	15	63 64	29 25	35	—	—	68	++	—
95	91	89	93	92	92	2,4,6-Trimethylphenol = mesitol	—	60	60	17	06	30	—	—	30 68	++	—
92	92	93	92	91	87	3,4,5-Trimethylphenol	—	08	08	64	62	—	—	—	71	+	—
95	90	92	92	93	92	2,6-Dibromo-4-*tert.*-butylphenol	—	08	62	60	59	+	—	—	71	—	—
95	93	92	90	89	92	2,4-Di-*tert.*-butylphenol	—	07	09	62	09	—	—	—	71	+	—
94	95	96	93	94	93	2,4,6-Tri-*tert.*-butylphenol	—	—	—	—	—	—	—	—	—	+	—
93	91	88	92	82	89	Dichlorophene = *o*-methylene-bis-*p*-chlorophenol	—	11	58	65	09	31 35	—	—	69	++	—
37	93	01	01	00	00	4-Chloro-*o*-anisidine	56	—	—	—	—	—	—	—	08	+	—
87	83	86	76	90	92	2-Chloro-4,6-dinitrophenol	56	—	—	+	—	—	—	+	—	+	++
93	93	96	96	95	95	5-Methoxyindole	71	03	+	+	10 62	24	—	—	51 69	+	—
94	95	94	96	96	96	5-Benzyloxyindole	—	—	—	—	09	23	—	—	71	+	—
94	91	96	92	93	89	*o*-Methoxybenzyl alcohol	—	—	—	—	—	—	—	—	—	+	—
84	83	84	37	22	03	*m*-Hydroxybenzyl alcohol	—	09	12	15	08	35	—	—	71	++	—
94	86	93	94	88	91	*m*-Methoxybenzyl alcohol	—	—	—	—	—	—	—	—	—	+	—
85	82	80	29	18	02	*p*-Hydroxybenzyl alcohol	—	08	10	15	07	35	—	—	71	++	—
91	88	84	87	83	81	*p*-Methoxybenzyl alcohol	—	—	—	—	—	—	—	—	—	+	—
94	92	92	88	87	86	2-Hydroxy-5-methylacetophenone	17	+	—	—	—	—	+	26 24	—	+	—
92	84	92	87	84	57	4-Hydroxy-3-methylacetophenone	—	09	—	+	06	—	57	—	—	+	—
95	93	92	89	90	90	*o*-Hydroxypropiophenone	—	07	09	+	+	40 23	+	24	—	+	—
93	90	92	89	87	68	*m*-Hydroxypropiophenone	—	09 13	60	60	11	39	+	—	—	+	—
90	87	84	86	79	49	5-Methoxy-3-indolealdehyde	34	—	—	—	—	—	08	—	71	+	—
92	18	89	86	77	45	Phenoxyacetic acid	—	—	—	—	—	—	—	58	—	—	++
85	05	69	25	09	00	*p*-Hydroxyphenoxyacetic acid	—	19	09	23 63	08 25	32 12	—	—	30	++	+
87	07	85	88	84	78	*o*-Methoxybenzoic acid	34	—	—	—	—	—	—	03	—	—	+
94	13	96	93	93	91	*m*-Methoxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+
93	66	94	90	89	74	3-Phenylsalicylic acid	38	10	60	65	09	38	—	24 68	—	++	++
90	34	93	61	66	08	5-Formylsalicylic acid	—	—	—	—	—	—	06	65	—	+	+
92	72	92	78	85	51	3,5-Dichlorosalicylic acid	38	07	57	64	06	—	—	23	—	++	++
93	78	91	85	87	64	3,5-Diiodosalicylic acid	69	—	—	—	—	—	—	24	—	+	++
84	63	79	42	46	06	3-Nitrosalicylic acid	56	—	—	—	—	03	—	63	06	—	+
93	04	90	73	66	04	3-Chloro-4-hydroxybenzoic acid	—	07	—	65	64	39	—	03	—	++	+
93	00	95	88	82	43	3-Nitro-4-hydroxybenzoic acid	56	07	07	—	12	—	—	57	09	+	+
93	73	95	93	91	85	*o*-Thymotic acid	38	12	08	56	08 23	29 25	—	70	56 71	+	+
89	08	83	39	19	00	3-Hydroxyanthranilic acid	38	58	57	62	63	42	—	59	51 68	+	+
89	04	90	53	38	02	*m*-Hydroxycinnamic acid	—	—	+	63	08 20	38	—	—	—	++	+
90	10	92	91	83	70	*p*-Methoxycinnamic acid	38	—	—	—	—	—	—	—	—	+	+
93	19	96	87	78	22	4-Hydroxy-α-phenylcinnamic acid	—	12	57	56 63	23	—	—	57	50 71	++	+
88	23	92	51	62	02	4,4′-Bis-(4-hydroxyphenyl)-pentanoic acid	—	09	11	15	20	38	—	—	69	++	+
88	08	93	80	58	12	5-Methoxy-2-carboxy-indole	33	—	+ 25	24	+	—	—	59	—	—	+

(continued on p. 465)

TABLE I (*continued*)

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
90	32	92	55	42	03	*o*-Hydroxyphenylacetic acid	34	12	14	65 63	08	29	—	71	51 30	++	+
88	05	86	48	35	02	*p*-Hydroxyphenylacetic acid	—	14	58	65	65 60	—	—	—	30	++	+
92	27	86	71	63	16	*o*-Hydroxyhydrocinnamic acid = melilotic acid	—	12	13	65	08	29	—	—	45	++	+
89	09	89	48	29	01	*p*-Hydroxycinnamic acid	38	07	12	65	08	26	—	60	30	++	+
87	22	78	31	13	00	*o*-Hydroxyphenyllactic acid	—	06	—	65	09	33	—	—	—	+	+
88	28	82	32	27	02	*o*-Hydroxyphenylpyruvic acid	34	08	09	65 62	09	41	06	48	71	+	++
90	19	80	11	08	01	*m*-Hydroxyphenylpyruvic acid	70	07	—	25 59	07	57	06	45	—	+	+
52	40	16	54	07	05	2-Hydroxypyridine	—	—	—	—	—	—	—	09	—	+	—
42	57	28	41	03	00	3-Hydroxypyridine	34	08	63 65	23	60 09	29	—	09	—	++	—
23	08	01	03	00	00	4-Hydroxypyridine	34	—	—	—	—	—	—	07	—	—	—

TABLE II

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,2-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
91	83	92	67	74	22	4-Chloropyrocatechol	—	10 14	14 64	17	56 65	29	—	42	68	++	—
93	92	91	89	89	74	3-Isopropylpyrocatechol	56	10 64	24	23	10 65	23	—	54 67	62 69	++	—
92	93	92	90	87	69	4-*tert.*-Butylpyrocatechol	56	05 60	27	27 71	08 64	24 28	—	03 48	62 25	++	—
92	93	90	92	89	87	3-Isopropyl-6-methylpyrocatechol	56	10 64	65 66	61	10 65	28 25	—	60	52 69	++	—
94	93	95	90	92	87	3,5-Diisopropylpyrocatechol	56	06 70	58 41	58 39	59	41 27	17	03	69 42	++	—
93	78	84	92	72	58	Veratryl alcohol	—	—	—	—	—	—	—	—	—	+	—
86	88	85	91	82	69	*m*-Acetovanillone	—	19	60	65	21 65	38	07	—	68	++	—
88	90	87	90	87	85	Zingerone = vanillyl-acetone	—	19	60	62	60 55	40	06	40 51	30	++	—
28	63	00	00	00	00	Vanillylamine	—	10	15	19	56 23	35	—	—	68	+	—
92	62	87	92	85	87	Ethylvanillin = 3-ethoxy-4-hydroxybenzaldehyde	—	09	—	61	59	+	08	—	71	++	—
94	79	93	92	93	90	5-Propenylvanillin	34	09	—	—	+	51	08	—	71	++	—
88	07	83	83	62	22	Opianic acid = 5,6-dimethoxy-2-formylbenzoic acid	26	—	—	—	—	—	03	—	—	+	+
92	14	86	88	80	02	Piperonylic acid	34	—	—	—	—	—	—	58	—	+	+
82	02	66	07	01	00	3,4-Dihydroxyphenylacetic acid	—	11	20	22	62	24	—	43	62 69	++	+
89	08	92	83	69	38	3-Ethoxy-4-hydroxycinnamic acid	38	65	23	25 54	38 43	25	—	57	69	++	+
91	12	90	90	89	67	2,3-Dimethoxycinnamic acid	32	—	—	—	—	—	—	—	—	+	+
82	00	69	45	04	00	Hemipinic acid	+	—	—	—	—	—	—	03	—	—	+

TABLE III

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,3-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
94	67	92	72	75	33	2,6-Dichlororesorcinol	—	09	64	65	10	23	—	70	68	++	—
93	92	92	87	91	82	2,5-Dimethylresorcinol	—	06	19	65	08	33	—	—	—	+	—
95	93	92	91	89	83	Dimethoxyorcinol	—	06	19	65	08	33	—	—	—	+	—
41	77	08	02	00	00	2-Aminoresorcinol	—	08 60	19	63	09	24	—	56	68	++	—
93	47	92	90	91	92	2-Nitroresorcinol	56	06 65	15	65	08	23	—	—	51	+	—
93	78	94	91	90	78	2,4-Dihydroxybenzophenone	71	64	20 63	23 25	11	28	+	62	05	+	—
94	93	95	92	93	93	2-Hydroxy-4-methoxybenzophenone	65	09	+	63	08	—	+	58	—	+	—
91	49	93	85	84	46	Orsellinic aldehyde	17	08	19	23	08	69	08	63	—	+	—
90	04	84	87	74	54	2,6-Dimethoxybenzoic acid	—	—	—	—	—	—	—	—	—	—	+
92	17	92	92	85	72	3,5-Dimethoxybenzoic acid	34	—	—	—	—	—	—	—	—	—	+
93	19	93	90	86	76	2,4-Dimethoxycinnamic acid	34	—	—	—	—	—	—	57	—	+	+

TABLE IV

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME 1,4-DIHYDRIC PHENOLS AND THEIR DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
86	dec.*	86	43	33	08	Methoxyquinol	34	52	56	51	10	66	—	60	49	++	—
78	00	65	32	21	08	2,5-Dihydroxy-1,4-benzoquinone	65	08	22	65	10	26	58	56	71	++	+
39	00	07	06	00	00	3,6-Dihydroxypyridazine	—	—	—	—	—	—	—	17	—	+	—
92	68	89	73	51	08	*p*-Dihydroxypropiophenone	34	58	63	—	61	52	58	43	09 68	++	—
92	92	95	94	92	93	2,5-Dimethoxybenzaldehyde	39	—	—	—	—	—	07	—	—	+	—
84	dec.*	71	03	02	00	Homogentisic acid	34	57	70	72	58	70	—	—	30 68	++	+
90	dec.*	90	78	71	26	Homogentisic lactone	+	24 52	62 69	15 27	63	23 45	—	—	25 68	++	—
95	12	94	92	92	91	2,5-Dimethoxyphenylacetic acid	—	—	—	—	—	—	—	—	—	+	+

* Decomposition.

4. RESULTS AND DISCUSSION

The first five tables (I–V) present chromatographic data on monohydric, 1,2-, 1,3-, 1,4-dihydric and trihydric phenols. The grouping into tables was the same as that previously used, in order to demonstrate the different colour reaction patterns, which are in accordance with earlier findings as far as the three groups of dihydric phenol derivatives are concerned. The 1,3-dihydric phenol derivatives show a very distinct colour pattern when the four diazonium reagents are applied, except when the OH-groups are methoxylated and, in addition, there is a carboxyl group present.

TABLE V

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME TRIHYDRIC PHENOLS

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
85	83	77	02	01	00	2,4,6-Trihydroxytoluene	+	10	63	65	10	24 59	—	56	68	++	—
54	16	18	00	00	00	2,3,6-Trihydroxybenzoic acid	—	07 60	23	21	08	62	—	03	50 69	+	+
83	01	78	77	35	08	3,5-Dimethoxy-4-hydroxybenzoic acid	+	08 15	21	35 20	15 35	33	—	—	68	++	+
89	08	88	90	79	69	3,4,5-Trimethoxycinnamic acid	32	—	—	—	—	—	—	07	—	++	+

TABLE VI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NAPHTHALENE AND QUINOLINE DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
93	93	93	90	86	86	4-Chloro-1-naphthol	30	14 21	23	25	15 61	35 28	—	24	25 28	++	—
55	93	00	00	00	00	1-Amino-4-naphthol	69	23	61	61	24	68	59	56	68	++	—
91	93	92	93	10	03	2-Methyl-4-amino-1-naphthol	71	23	+	+	23	—	—	05	50 30	++	—
82	87	87	59	27	04	1-Amino-7-naphthol	33	22 65	25	28	28 23	23	—	69	24 69	+	—
77	85	75	56	21	04	2-Amino-7-naphthol	69	10	19	23	19 24	68	60	56	68	++	—
88	87	85	82	62	11	2-Amino-3-naphthol	30	10 64	23	24	19 68	42	—	+	68	+	—
94	57	94	92	92	75	2-Hydroxy-1-naphthoic acid	34	11	23 65	24 25	12	45	17	69	69	++	++
92	56	93	84	84	54	3-Hydroxy-2-naphthoic acid	03	12 15	25	25 28	15	45	—	28 68	71	++	++
93	85	93	92	93	94	5-Hydroxy-1,4-naphthoquinone = juglone	56	—	—	—	—	—	+	+	—	+	—
91	79	92	74	68	19	2,3-Dihydroxynaphthalene	—	62 63	23 25	25 68	19	51	—	29	68	++	—
91	05	87	92	90	92	5,8-Dihydroxy-1,4-naphthoquinone = naphthazarin	56	71	69	24	03	26	17	71	71	—	—
83	76	69	82	58	52	2-Hydroxyquinoline	30	—	—	—	—	—	—	—	—	+	—
71	58	33	45	03	00	4-Hydroxyquinoline	34	—	—	—	—	—	—	13	—	—	—
58	76	54	69	09	01	5-Hydroxyquinoline	69	08 14	65 23	65 25	10	35	01	51	07 69	++	—
73	89	78	90	68	67	8-Hydroxyquinoline	57	14	15	23	63	35	—	39	69	+	—
68	55	35	69	03	00	4-Hydroxyquinaldine	—	—	—	—	—	—	—	08	—	—	—
57	92	62	93	56	47	8-Hydroxyquinaldine	—	12 21	15	65 63	12 61	30 41	—	70	68	+	—
74	02	49	dec.[*]	00	00	2,4-Dihydroxyquinoline	34	06	19	23	08	69	—	—	—	+	—
59	00	15	00	00	00	4,8-Dihydroxyquinoline-2-carboxylic acid = xanthurenic acid	34	17	—	—	17	40	—	—	—	—	+
51	28	16	17	00	00	2,3-Dihydroxyquinoxaline	32	—	—	—	—	—	—	—	—	—	—

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[*] Decomposition.

TABLE VII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BENZOIC AND PHTHALIC ACID DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
90	33	91	89	79	39	Benzoylformic acid	34	08	11	63	09	38	06	—	71	+	+
93	93	92	89	90	90	*p*-Chlorobenzaldehyde	—	—	—	—	—	—	06	—	—	+	—
91	39	87	88	87	83	2,4-Dichlorobenzoic acid	—	—	—	+	—	—	—	—	—	—	++
93	53	95	88	88	72	3,5-Dichloroanthranilic acid	38	—	—	—	—	—	—	—	—	+	+
92	05	88	49	37	06	2-Chloro-4-aminobenzoic acid	—	—	—	+	07	—	—	57	—	+	+
90	19	89	89	82	33	2-Acetamidobenzoic acid	30	—	—	—	—	—	—	04	—	—	+
86	03	83	43	09	01	4-Acetamidobenzoic acid	—	—	—	—	—	—	—	—	—	—	+
32	02	02	00	00	00	2,5-Diaminobenzoic acid	71	—	60	—	59	26 63	—	55	44 70	++	+
41	00	11	02	00	00	3,5-Diaminobenzoic acid	38	12	65	23	15	39 23	—	59	—	++	+
92	48	89	81	84	48	3,5-Dinitrobenzoic acid	65	—	—	—	—	—	—	—	—	—	+
90	12	92	84	69	28	*o*-Carboxybenzaldehyde	—	—	—	—	—	—	01	—	—	+	+
94	93	92	96	95	95	Terephthalaldehyde	—	—	—	—	—	—	06	—	—	+	—
92	00	87	64	35	00	5-Methylisophthalic acid = uvitic acid	—	—	—	—	—	—	—	—	—	—	+
86	00	68	05	00	00	5-Aminoisophthalic acid	38	—	+	+	06	—	—	58	—	+	+
92	00	92	38	29	00	5-Nitroisophthalic acid	34	—	—	—	—	—	—	—	—	—	+
88	00	79	31	17	00	2-Nitroterephthalic acid	71	—	—	—	—	—	—	—	—	—	++
92	02	89	80	59	00	Diphenic acid = diphenyl-2,2′-dicarboxylic acid	—	+	—	+	—	—	—	—	—	—	++
57	00	02	00	00	00	Prehnitic acid	—	—	—	—	—	—	—	—	—	—	++

In that case the compounds do not react with the diazonium reagents and dibromoquinonechloroimide.

In general, the R_F values seem to follow the same pattern as found earlier, although the compounds given in the present tables are more heterogeneous, and should be regarded together with the values obtained before. The introduction of an amino group gives a maximum value for solvent E, the R_F values in all other solvents being lowered considerably, even down to 00 in solvents C and D. The presence of a free carboxyl group is demonstrated by a low value in solvent E and this is significant. This holds also for monohydric phenols. It is interesting to note the behaviour of the monohydroxypyridines (Table I) in the different solvent systems. The second maximum value in solvent B, besides that in F or E, was not found before for any of the types of compounds investigated by this procedure. Hydroxyquinolines and hydroxyquinaldines (Table VI) behave similarly. Also all the alkaloids in Table XIII show the same type of R_F pattern in these solvents.

The limited space allows no detailed discussion here. It is hoped to present the condensed results of the two paper-chromatographic studies together later on.

Table IX gives data for pyridinedicarboxylic acids; no colour reactions and zero values for R_F in solvents E, C and D remind one of the values obtained for the three phthalic acids. In Tables X to XIV many compounds of metabolic origin are found. The derivatives of aromatic amino acids are only practically separated in solvent F and this could therefore be regarded as significant for this type of compound. When

TABLE VIII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC NON-PHENOLIC AND HETEROCYCLIC MONOCARBOXYLIC ACIDS WITH THE COOH GROUP IN THE SIDE CHAIN, AND THEIR DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
90	29	87	92	89	88	α-Methylcinnamic acid	—	—	—	—	+	—	—	03	71	++	+
92	22	90	90	89	83	2-Chlorocinnamic acid	—	—	—	+	—	—	—	—	—	+	+
92	18	90	91	88	86	3-Chlorocinnamic acid	—	—	—	+	—	—	—	—	—	+	+
94	20	95	90	88	65	3-Nitrocinnamic acid	65	—	—	—	—	—	—	—	—	+	+
90	28	88	67	61	12	*p*-Chloromandelic acid	—	—	—	—	—	—	—	04	—	+	+
86	08	81	72	36	06	*dl*-Tropic acid	—	—	—	—	—	—	—	—	—	+	+
87	28	82	78	67	22	*dl*-Atrolactic acid	—	—	—	—	—	—	—	—	—	+	+
94	33	92	92	92	91	2-Phenyl-*n*-butyric acid	—	—	—	—	—	—	—	—	—	—	+
93	48	89	89	89	87	Diphenylacetic acid	—	—	—	+	—	—	—	—	+	—	+
49	00	27	34	02	00	*p*-Aminophenylacetic acid	48	59	—	10	07	—	—	—	+	+	+
92	92	85	92	91	92	*p*-Nitrophenylacetic acid	71	—	—	—	—	03 40	—	—	—	—	+
87	07	83	71	54	18	2-Furoic acid	34	—	—	—	—	—	—	03	—	+	+
89	13	88	89	83	74	β-(2-Furyl)-acrylic acid	57	—	56	25	07	—	—	60	71	++	+
92	19	86	82	79	62	2-Thiophenecarboxylic acid	—	—	—	—	—	—	—	03	—	+	+
11	09	00	00	00	00	4-Imidazolecarboxylic acid	34	08	—	63	07	—	02	—	—	+	+
24	01	02	01	00	00	Imidazolyl-4 (or 5)-acrylic acid = urocanic acid	—	07	17 58	20 65	09	—	—	+	—	++	+
08	21	05	04	00	00	Imidazolylpyruvic acid	33	61	60	23	60	—	+	51	69	+	+
90	88	93	89	79	50	3-Indolealdehyde	34	—	—	—	—	—	08	—	71	+	—
84	05	82	42	18	02	*dl*-2-(3-Indolyl)-lactic acid	—	—	—	—	—	—	—	—	—	+	+
46	05	16	28	00	00	Isonicotinic acid	—	—	—	—	—	—	—	—	—	—	+
29	02	09	47	03	01	6-Methylpicolinic acid	—	—	—	—	—	—	—	—	—	—	+
95	07	64	82	00	00	6-Quinolinecarboxylic acid	—	—	—	—	—	—	—	—	—	—	+

TABLE IX

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF PYRIDINEDICARBOXYLIC ACIDS

R_F values × 100						*Compounds*	*Detection** *Ind*
F	*E*	*A*	*B*	*C*	*D*		
45	00	03	00	00	00	2,3-Pyridinedicarboxylic acid = quinolinic acid	+
58	00	11	00	00	00	2,4-Pyridinedicarboxylic acid = lutidinic acid	+
54	00	15	09	00	00	2,5-Pyridinedicarboxylic acid = isocinchomeronic acid	+
96	00	41	33	06	00	2,6-Pyridinedicarboxylic acid = dipicolinic acid	+
40	00	02	00	00	00	3,4-Pyridinedicarboxylic acid = cinchomeronic acid	+
77	00	43	13	00	00	3,5-Pyridinedicarboxylic acid = dinicotinic acid	+

* The compounds listed in this table gave no reaction with the reagents used for detection in the previous tables, except Ind.

the amino group is acetylated the compounds behave in general like carboxylic acids. One example of this is acetyltryptophan, and more examples are to be found in Table XIV, where the hippuric acids are tabulated. Although the structure of these compounds is rather similar, the only differences being the presence of other substituents in the aromatic part of the molecule, chromatographic separation is limited to three solvents, A, B and C. The difficulty in distinguishing between several struc-

TABLE X

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME AROMATIC, HETEROCYCLIC AND ALIPHATIC AMINO ACID DERIVATIVES

R_F values × 100						Compounds	Detection										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*NH**	*Fe*	*Mo*	*Mn*	*Ind*
42	07	02	02	00	00	DL-*p*-Chlorophenylalanine	—	—	—	—	—	—	23	—	71	+	—
30	04	01	00	00	00	*o*-Fluorophenylalanine	—	—	—	—	06	+	23	—	—	—	—
29	05	01	00	00	00	*m*-Fluorophenylalanine	—	—	—	—	07	+	23	—	—	—	—
31	05	01	00	00	00	*p*-Fluorophenylalanine	—	—	—	—	06	—	23	—	—	—	—
44	04	02	00	00	00	DL-Thyronine	—	14	—	63	09	35	23	—	68	++	—
00	05	00	00	00	00	DL-Thyroxine	—	06	—	—	62	—	58	—	—	+	—
00	00	00	00	00	00	3-Aminotyrosine	34	10 59	62	56	11 56	59	23	23	68	++	—
83	03	81	53	11	01	DL-Acetyltryptophan	—	—	—	—	07	+	—	—	—	++	+
35	03	01	00	00	00	DL-4-Methyltryptophan	—	—	—	+	08	22	23	—	71	+	—
27	00	00	00	00	00	DL-4-Hydroxytryptophan	34	—	+	24	—	26 71	23	—	71	+	—
37	03	00	00	00	00	DL-5-Methyltryptophan	—	—	—	59	07	—	23	—	—	+	—
13	01	00	00	00	00	DL-5-Hydroxytryptophan	—	19 62	65	25	63	35	+	+	68	++	—
27	02	00	00	00	00	Kynurenine	40	—	—	—	—	—	23	—	—	+	—
22	00	00	00	00	00	3-Hydroxykynurenine	48	14	23 65	63	14 24	41	59	62	51 42	+	—
00	00	00	00	00	00	Histidine	34	13	11	15 62	07	—	24	—	—	+	—
00	02	00	00	00	00	DL-1-Methylhistidine	71	+	57	60	07	—	23	—	—	+	—
04	28	00	00	00	00	6-Aminonicotinamide	—	—	—	—	06	—	—	—	06 72	+	—
48	02	11	01	00	00	6-Hydroxynicotinic acid	—	—	—	—	—	—	—	—	58	+	+
10	01	00	00	00	00	DL-Pipecolic acid	—	—	—	10	06	—	26	—	—	—	+
17	01	00	00	00	00	α-Aminophenylacetic acid	—	—	—	—	—	—	—	—	—	—	+
90	00	91	52	26	00	N-*o*-Carboxyphenyl-glycine	41	07	—	08	10	41	—	52	71	++	+
87	81	04	05	00	00	N-*p*-Hydroxyphenyl-glycine	—	—	—	+	—	—	—	—	+	—	+
06	00	00	00	00	00	DL-2-(3,4-Dihydroxyphenyl)-glycine	—	17	19	17	07	51 71	—	42	68	+	—
73	01	33	03	00	00	*p*-Aminobenzoyl-L-glutamic acid	57	—	—	+	06	—	58	—	+	++	++
84	09	80	86	42	10	ω-Benzoylamino-*n*-valeric acid	—	—	—	—	—	—	—	—	—	—	+
85	11	83	88	60	18	ω-Benzoylamino-*n*-caproic acid	—	—	—	—	—	—	—	—	—	—	+
47	00	07	05	00	00	Formylglycine	34	—	—	—	—	—	24	—	—	—	++
02	00	00	00	00	00	Sarcosine	—	—	—	08 72	06	59	24	57	—	+	—
54	00	08	14	00	00	Aceturic acid	—	—	—	—	—	—	23	—	—	—	++
35	00	01	00	00	00	Ureidosuccinic acid	—	—	—	—	—	—	26	—	—	—	+

* NH = ninhydrin reagent.
DN reagent gave no reaction with the compounds listed in this table.

tures is somewhat lessened by the use of several reagents for detecting hippuric acids, as pointed out in the section on additional reagents. Although the shades produced by these reagents are rather similar, the time within which they develop is in some cases decisive.

The main characteristic of biologically active nitrogen compounds of amine type is their high R_F value in E, and also values other than 0 in F. For indole derivatives no generalization can be made, since it depends on the substituents to which type they belong; in the case of OH- substituents, or only alkyl residues, they belong

TABLE XI

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME BIOLOGICALLY ACTIVE NITROGEN COMPOUNDS

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
41	85	02	04	00	00	*β*-Phenylethylamine	—	—	—	08	06	—	—	—	—	—	—
19	88	00	00	00	00	*dl*-*β*-Hydroxyphenylethylamine	—	—	—	59	—	—	—	—	—	+	—
39	88	02	12	00	00	*d*-Amphetamine	—	—	—	+	—	—	—	—	—	—	—
17	dec.*	00	00	00	00	3-Hydroxythyramine = DOPA-amine	—	15	20	21	60	25	—	45	68	++	—
18	26	00	00	00	00	3,4-Dihydroxynorephedrine = corbasil	—	17	17	17	65	26	—	51	69	+	—
40	85	00	09	00	00	Hordenine	—	13	60	62	07	26 64	—	—	—	++	—
08	02	00	02	00	00	Mescaline	—	—	—	—	—	—	—	—	—	+	—
01	28	00	00	00	00	Histamine	—	09	—	64 10	64	—	—	—	—	—	—
05	00	00	00	00	00	Trigonelline	—	—	—	—	—	—	—	57	—	—	—
95	92	96	96	94	95	2-Methylindole	03	07	+	62	11	24	—	—	+	+	—
94	96	93	96	93	93	5-Methylindole	—	—	—	—	09	24	—	—	71	+	—
44	87	03	00	00	00	Tryptamine	—	—	—	08	07	23	—	—	—	++	—
36	83	01	00	00	00	Bufotenine	—	17	65 23	23	62	37	—	—	69	+	—
28	12	07	12	00	00	Adrenochrome	57	—	—	—	—	70	—	—	71	+	—
71	88	07	64	01	00	Lysergic acid diethylamide tartrate	33	—	—	—	—	—	—	—	—	—	—
27	32	01	02	00	00	4-Desoxypyridoxine	—	11	+	25	12 19	35	—	62	71	+	—
02	53	00	00	00	00	Pyridoxamine	33	08 11	60 65	63 24	12	38 35	—	62	71	+	—
28	23	02	02	00	00	4-Pyridoxic acid	32	08	+	23	09	38	—	58	—	+	+
02	00	00	00	00	00	Pyridoxal-5-phosphate	34	—	—	+	10	39	01	—	—	—	+
18	03	00	01	00	00	Creatinine	—	—	—	—	—	—	—	58	+	—	—
09	00	00	00	00	00	Riboflavine-5′-phosphate	03	—	—	—	—	—	—	—	—	+	++
30	00	02	01	00	00	Hydantoic acid**	—	—	—	—	—	—	—	—	—	—	+
33	23	05	06	00	00	Hydantoin**	—	—	—	—	—	—	—	—	—	—	—
93	91	97	97	93	92	Alloxan	—	—	—	—	—	—	—	—	—	—	33
27	11	02	00	06	00	Allantoic acid**	—	—	—	—	—	—	—	—	—	—	+
12	02	00	00	00	00	Allantoin**	—	—	—	—	—	—	—	—	—	—	—
94	03	00	00	00	00	Dialuric acid = 5-hydroxybarbituric acid	—	—	—	—	—	—	—	—	30	—	+
78	04	00	00	00	00	Parabanic acid	—	—	—	—	—	—	—	—	—	—	+
02	00	00	00	00	00	Betaine**	—	—	—	—	—	—	—	—	—	—	—
25	09	01	00	00	00	Urea**	—	—	—	—	—	—	—	—	—	—	—
03	00	00	00	00	00	Aneurine	—	—	60	60	—	—	—	—	71	+	—

* Decomposition. ** These compounds were detected by the EH and DAC reagents.

to the phenol type, giving violet-red colours with dibromoquinonechloroimide reagent. The presence of a carboxyl group gives them the character of an aromatic carboxylic acid with a side chain, and they can only be distinguished from the latter by the positive reaction with the Ehrlich reagent. This reagent is by no means selective in the same sense as the hippuric acid reagent is for hippuric acids. Some amino acid and carbamoyl derivatives produce yellow to orange colours, as has been shown by the examples given in the section on additional reagents. Also some pyrrolecarboxylic acids, urea, kynurenine and anthranilic acid derivatives give strong colours and this

TABLE XII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME INDOLE DERIVATIVES

RF values × 100						*Compounds*	*Detection*														
F	*E*	*A*	*B*	*C*	*D*		*EH*	*U.V. EH*	*DAC*	*U.V. DAC*	*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
92	90	94	93	94	92	Indole	24	—	28 33	—	—	06	—	—	10	25	15	—	03	+	—
95	92	96	96	94	95	2-Methylindole	22	65	35 25	23	03	07	+	62	11	24	—	—	+	+	—
94	96	93	96	93	93	5-Methylindole	22	—	23 28	45	—	—	—	—	09	24	—	—	71	+	—
95	92	90	92	92	92	Indoxyl acetate	57	—	23	23	38	+	—	—	60	+	—	—	71	+	—
93	93	96	96	95	95	5-Methoxyindole	24 20	—	38 28	—	71	03	+	+	10 62	24	—	—	51 69	+	—
94	95	94	96	96	96	5-Benzyloxyindole	18 22	—	40 25	—	—	—	—	—	09	23	—	—	71	+	—
92	00	91	84	67	28	Isatin	—	—	62	—	65	07	08	60	—	—	03	—	—	+	—
90	88	93	89	79	50	3-Indolealdehyde	26	—	22 63	—	34	—	—	—	—	—	08	—	71	+	—
90	87	84	86	79	49	5-Methoxy-3-indolealdehyde	26	—	26	—	34	—	—	—	—	—	08	—	71	+	—
87	14	90	73	65	23	α-(3-Indolyl)-acetic acid	60 70	—	22	—	—	—	—	03	07	58 17	—	—	—	++	+
92	15	92	83	72	42	β-(3-Indolyl)-propionic acid	23 71	—	23	—	—	—	—	—	08	—	—	—	—	+	+
94	17	93	88	83	50	γ-(3-Indolyl)-butyric acid	23 70	—	23	—	—	—	—	—	08	—	—	—	—	+	+
84	05	82	42	18	02	DL-2-(3-Indolyl)-lactic acid	26 71	—	25	64	—	—	—	—	—	—	—	—	—	+	+
75	00	49	21	03	00	DL-2-(3-Indolyl)-lactic acid, double spot	26	—	22	—	—	—	—	—	—	—	—	—	—	+	+

(continued on p. 473)

TABLE XII (*continued*)

R_F values × 100						Compounds	Detection														
F	*E*	*A*	*B*	*C*	*D*		*EH*	*U.V. EH*	*DAC*	*U.V. DAC*	*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
88	08	93	80	58	12	5-Methoxy-2-carboxy-indole	26 25	—	70	—	33	—	25	24	+	—	—	59	—	—	+
92	03	80	07	04	00	5-Hydroxy-3-indoleacetic acid	68	—	24	68	03	14	65	25	62	68	—	—	30	++	+
36	03	00	00	00	00	L-Tryptophan	57	—	23	—	—	—	—	—	06	—	—	—	—	+	—
83	03	81	53	11	01	DL-Acetyltryptophan	60 26	63	25 22	20	—	—	—	—	07	+	—	—	—	++	—
35	03	01	00	00	00	DL-4-Methyltryptophan	—	—	23	—	—	—	—	+	08	22	—	—	71	+	—
27	00	00	00	00	00	DL-4-Hydroxytryptophan	71	—	26 52	65	34	—	+	24	—	26 71	—	—	71	+	—
37	03	00	00	00	00	DL-5-Methyltryptophan	—	—	23 25	—	—	—	—	59	07	—	—	—	—	+	—
13	01	00	00	00	00	DL-5-Hydroxytryptophan	—	—	26	—	—	19 62	65	25	63	35	—	+	68	++	—
44	87	03	00	00	00	Tryptamine	70 51	—	23	—	—	—	—	08	07	23	—	—	—	++	—
53	84	01	09	00	01	Gramine	57	66	22	65	—	—	07	07	11	24	—	—	03	+	—
38	64	00	00	00	00	Serotonine	71	—	26	—	65	20	24	25	63	35	—	70	28	+	—
36	83	01	00	00	00	Bufotenine	27 71	70	30 25	19	—	17	65 23	23	62	37	—	—	69	+	—
28	12	07	12	00	00	Adrenochrome	—	—	—	—	57	—	—	—	—	70	—	—	71	+	—
71	88	07	64	01	00	Lysergic acid diethyl-amide tartrate	24	—	24	—	33	—	—	—	—	—	—	—	—	—	—
86	95	44	91	12	05	Reserpine*	—	—	—	—	34	—	—	—	—	—	—	—	—	—	—
57	85	02	23	00	00	Yohimbine*	—	—	—	—	34	—	—	—	—	—	—	—	—	—	—
48	92	03	42	03	03	Physostigmine*	—	—	—	—	—	—	—	09	06	—	—	—	71	+	—
32	67	02	75	03	01	Brucine*	—	—	—	—	—	—	—	—	—	—	—	—	—	+	—
43	83	02	65	03	01	Strychnine*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* Also detected by Bi reagent.

TABLE XIII

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME NATURAL PRODUCTS OF VEGETABLE AND MOULD ORIGIN

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
75	72	55	89	60	55	2,6-Dimethyl-γ-pyrone	—	—	—	64	—	—	—	+	—	+	—
89	07	82	86	84	80	Coumarin-3-carboxylic acid	34	—	—	—	—	—	—	—	—	+	+
89	55	84	43	13	00	5,7-Dihydroxy-4-methylcoumarin	33	12	66	65	12	35	—	—	03 33	+	—
76	03	23	05	00	00	Quercitrin	56	07	15 63	62	58	47	—	50	08 51	++	—
84	67	58	00	00	00	*d*-Catechin	—	10	65	65	11	24	—	51	09 42	++	—
37	13	01	00	00	00	Neohesperidin	—	07	11	63	08 65	33 35	—	57 56	71	+	—
88	06	73	33	05	00	Gibberellic acid	—	—	—	—	—	—	—	—	—	+	+
13	76	00	03	00	00	Cinchonine*	34	—	—	—	—	56	—	—	—	72	—
12	77	00	00	00	00	Morphine*	—	13	58	60	57	52	—	71	30	++	—
47	91	00	14	00	00	Atropine*	—	—	—	—	—	—	—	—	—	—	—
57	85	02	23	00	00	Yohimbine*	34	—	—	—	—	—	—	—	—	+	—
86	95	44	91	12	05	Reserpine*	34	—	—	—	—	—	—	—	—	—	—
38	76	02	30	00	00	Pilocarpine*	—	—	—	—	—	—	—	—	—	—	—
48	92	03	42	03	03	Physostigmine*	—	—	—	09	06	—	—	—	71	+	—
32	67	02	75	03	01	Brucine*	—	—	—	—	—	—	—	—	—	+	—
43	83	02	65	03	01	Strychnine*	—	—	—	—	—	—	—	—	—	—	—
37	01	03	00	00	00	Isoascorbic acid	34	72	—	—	+	72	—	72	38	++	+
92	45	92	61	43	00	Alternariol	38	62	23	65	08	25	—	71	—	+	—
92	85	88	88	87	42	Alternariol monomethyl ether	38	60	+	65	—	24	—	—	—	+	—
92	06	86	92	23	06	Stipitatonic acid	48	08	63	22 65	11	56	72	58	—	+	—
04	02	00	00	00	00	Aureomycin	03	+	+	17	08	70 56	—	58	—	—	—
39	02	07	07	00	00	Terramycin	03	—	—	07 69	11	26	—	57	—	—	—
90	91	86	71	42	06	Chloromycetin = chloramphenicol	65	—	—	—	—	—	—	—	—	+	—

* Also detected with Bi reagent.

TABLE XIV

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME HIPPURIC ACID DERIVATIVES

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DAB**	*Fe*	*Mo*	*Mn*	*Ind*
86	07	70	58	12	00	Hippuric acid	—	—	—	—	—	—	08	—	—	—	+
88	14	85	82	47	04	L-Benzoylalanine	—	—	—	—	—	—	+	—	—	—	+
78	05	38	15	02	00	α-Hydroxyhippuric acid	—	—	—	—	—	—	+	—	—	—	+
92	05	86	56	32	02	*o*-Hydroxyhippuric acid	38	06	—	+	08	38	08 17	23	—	+	+
78	06	53	10	00	00	*m*-Hydroxyhippuric acid	—	07	—	+	+	38	06 17	—	—	+	+
79	04	44	04	00	00	*p*-Hydroxyhippuric acid	—	+	—	+	60	—	08	—	—	+	+
84	07	72	83	31	06	*o*-Methoxyhippuric acid	—	—	—	—	—	41	07	—	—	—	+
83	08	67	65	13	01	*p*-Methoxyhippuric acid	—	—	—	—	—	—	09	—	—	—	+
84	12	72	67	27	03	*o*-Chlorohippuric acid	—	—	—	—	—	—	08 13	—	—	—	+

(Continued on p. 475)

TABLE XIV (*continued*)

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DAB**	*Fe*	*Mo*	*Mn*	*Ind*
88	11	84	68	30	04	*o*-Iodohippuric acid	—	—	—	—	—	—	+	—	—	—	+
69	03	24	07	00	00	*p*-Aminohippuric acid	34	—	—	63	08	—	07 11	+	+	+	+
91	10	77	53	20	01	*m*-Nitrohippuric acid	65	—	—	—	—	—	08 13	—	—	—	+
88	12	82	57	18	01	*p*-Nitrohippuric acid	56	—	—	—	—	—	62 65	—	—	—	+
91	17	87	48	36	02	3,5-Dinitrohippuric acid	65	—	—	—	—	—	62	—	—	—	+
68	01	18	00	00	00	3,4-Dihydroxyhippuric acid	—	—	—	—	07	—	+	42	71	+	+
89	09	78	77	27	02	Cinnamoylglycine	—	—	—	64	—	—	08 17	—	—	++	+
85	08	70	69	15	01	Phenaceturic acid	—	—	—	—	—	—	—	—	—	—	+
85	04	70	32	08	00	*o*-Hydroxyphenaceturic acid	—	08	11	65 63	23 19	33 28	22	+	71	+	+
92	10	52	34	02	00	Phenacetylglutamic acid	—	—	—	—	—	—	—	—	—	—	+
88	02	75	80	33	03	Phenoxyaceturic acid	—	—	—	—	—	—	—	—	—	—	+
49	02	08	12	00	00	Nicotinuric acid	—	—	—	—	—	—	08 17	—	—	—	+
43	00	03	00	00	00	Phthaloylglycine	—	—	—	—	—	—	11	—	—	—	+

* DAB = hippuric acid reagent. DN reagent gave no reaction with the compounds listed in this table.

TABLE XV

PAPER-CHROMATOGRAPHIC SEPARATION AND IDENTIFICATION OF SOME MISCELLANEOUS ALIPHATIC ACIDS

R_F values × 100						*Compounds*	*Detection*										
F	*E*	*A*	*B*	*C*	*D*		*U.V.*	*D1*	*D2*	*D3*	*D4*	*DB*	*DN*	*Fe*	*Mo*	*Mn*	*Ind*
27	01	02	10	00	00	Glyoxylic acid	—	—	—	—	—	—	+	—	—	+	++
70	02	37	28	04	00	β-Hydroxybutyric acid	—	—	—	—	—	—	—	—	—	+	++
76	10	31	09	00	00	β-Hydroxy-β-methylglutaric acid	—	—	—	—	—	—	—	—	—	23	+
91	16	92	93	88	88	DL-Thioctic acid	—	—	—	—	—	52	—	—	—	++	+
82	00	61	19	03	00	Thiomalic acid	—	— 72	— 72	— 72	— 72	60	—	38 72	49 30	++	++
40	00	18	06	00	00	Dihydroxyfumaric acid	57	72	72	09 72	05	72	—	72	47 30	++	++
85	00	77	61	17	00	3-Methyladipic acid	—	—	—	—	—	—	—	—	—	—	+
89	01	82	73	28	02	3,3-Dimethylglutaric acid	—	—	—	—	—	—	—	—	—	—	++
90	00	91	88	67	00	Sebacic acid	—	—	—	—	—	—	—	—	—	—	+

should be borne in mind when dealing with indole derivatives. The modified reagent, which was prepared on the basis of *p*-dimethylaminocinnamaldehyde in hydrochloric acid has some advantage over the common Ehrlich reagent, since the colours are in most cases stronger and red and blue shades dominate, which sometimes makes it possible to discern the spots more easily.

ACKNOWLEDGEMENTS

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SUMMARY

Supplementary data are given for the paper-chromatographic investigation of mono-, di- and trihydric phenol derivatives and various compounds of biochemical interest, such as biogenic amines, aromatic and heterocyclic amino acids, hippuric acids, indole derivatives. Some further types of compounds such as alkaloids, hydroxylated quinoline and pyridine derivatives and antibiotics, not dealt with in previous investigations reported earlier in this journal, have now been included. The R_F values of these compounds in six different solvent systems together with the corresponding colour reactions produced by ten standard reagents have been determined and are presented in 15 tables. Altogether approx. 270 compounds have been investigated and 20 hippuric acid derivatives synthetized. Application of these procedures to the clinical-chemical analysis of urine samples in order to detect metabolic excretion or detoxification products is indicated.

The hippuric acid reagent was modified so that it contained *p*-dimethylaminocinnamaldehyde in acetic acid anhydride. The Ehrlich reagent was prepared on the basis of *p*-dimethylaminocinnamaldehyde in hydrochloric acid; the colours produced with this reagent made it possible to differentiate the indole derivatives more distinctly.

The relation between the chemical structure of the compounds studied and the R_F values and colours produced with the detection reagents, is discussed.

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INORGANIC ADSORPTION PAPER CHROMATOGRAPHY

II. THE ADSORPTION OF HYDROGEN IONS BY FILTER PAPER

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The primary process in the separation of inorganic compounds by paper chromatography is usually the partition of the compound between two liquid phases, but many factors, including adsorption can be involved. The assessment of the relative importance of this adsorption factor requires a knowledge of the mechanism involved and the adsorptive capacity of the paper.

This information is not available in the literature although some studies have been made of the adsorption of acids and metal ions by cellulose products other than filter paper[1–11].

A quantitative study has therefore been made of the adsorptive power of the paper for hydrogen ions and other selected cations. The results of the first of these studies are recorded in this article.

PROCEDURE

Multiple 1-g samples of Whatman No. 1 filter paper were allowed to come to equilibrium with 20 ml of dilute nitric acid solution (10^{-4} to 10^{-2} M) contained in clean dry weighing bottles. To compensate for any tendency of the glass to adsorb hydrogen ions, acid blanks were retained in similar vessels. The increase in pH value of the acid solution due to hydrogen ion adsorption by the paper was accurately measured with a Cambridge Precision pH-meter. The amount of hydrogen ion adsorbed per gram of dry paper was then calculated from the change in pH of the acid solutions. (Allowance was made for the moisture content of the paper used.) The adsorptive capacity of the paper was confirmed by using a titration procedure similar to that reported by SOOKNE AND HARRIS[5].

In a second series of tests, the concentration of alkali salts liberated from the paper by acid treatment was determined. One-gram samples of paper were treated with 20 ml of either distilled water or dilute nitric acid solution and allowed to come to equilibrium. The sodium and potassium content of these solutions was then determined with the aid of a Lange flame photometer. The combined alkaline earth content was estimated by titrating an aliquot of the solution in the presence of an ammoniacal buffer with a mM solution of disodium ethylenediaminetetraacetate.

Comparative tests were made using Whatman No. 40 filter paper, viscose rayon fibre and cellulose acetate fibre.

The results of these studies are recorded in Tables I and II. To facilitate discussion these results have been related to the ash content of the cellulosic material in Table III.

TABLE I

ADSORPTION OF HYDROGEN IONS BY CELLULOSE PRODUCTS

μg of hydrogen ion adsorbed per g of dry adsorbent.

Cellulose product	*Concentration of HNO_3 in acid solution*				
	10^{-4} *M*	$5 \cdot 10^{-4}$ *M*	10^{-3} *M*	$5 \cdot 10^{-3}$ *M*	10^{-2} *M*
Whatman No. 40 filter paper	—	3.2	2.7	3.1	(3.1)*
Cellulose acetate fibre	—	5.4	8.5	10.8	9.7
Whatman No. 1 filter paper	2.7	7.1	10.7	11.0	10.7 (10.5)*
Viscose rayon fibre	1.1	—	14.8	24.4	26.1 (25.8)**

* Confirmatory results obtained by a titration technique.

** Confirmatory results obtained with $5 \cdot 10^{-2}$ *M* acid.

TABLE II

CONCENTRATION OF ALKALI SALTS LIBERATED BY ACID TREATMENT

Moles ($\times 10^{-6}$) of salt liberated per g of dry adsorbent.

Cellulose product	*Displacing solvent*	Na^+	K^+	$Ca^{+2} + Mg^{+2}$
Whatman No. 40 filter paper	Water	1.1	None detected	0.7
	Nitric acid	0.9		1.6
Cellulose acetate fibre	Nitric acid	10.7	None detected	2.6
Whatman No. 1 filter paper	Water	4.3	0.7	1.5
	Nitric acid	4.1	0.6	5.7
Viscose rayon fibre	Nitric acid	44	9.6	0.8

TABLE III

THE ADSORPTION OF HYDROGEN IONS AND DISPLACEMENT OF ALKALI SALTS THROUGH ACID TREATMENT OF CELLULOSE PRODUCTS

Cellulose product	*Approx. ash content %*	*Adsorptive capacity for H^+ $\mu g/g$*	*μg ions of cation liberated/g by acid*	
			Na + K + Ca + Mg	*(Ca + Mg) × 2*
Whatman No. 40 filter paper	0.01	3.1	2.6	3.2
Cellulose acetate fibre	0.06	10.2	13.3	5.2
Whatman No. 1 filter paper	0.06	10.8	10.4	11.2
Viscose rayon fibre	0.20	26.0	64	1.6

DISCUSSION

It can be seen from Tables I and III that each cellulose product has an adsorptive capacity for hydrogen ions which is related to the ash content of the product. A similar observation was made by EDGE[2] in his study of the adsorption of acids by paper pulp solutions.

If allowance is made for experimental error, reference to Table III shows that the adsorptive capacity of the two filter papers (estimated in terms of micro-equivalents of hydrogen) is equal to either

(1) the total number of μg ions of sodium, potassium, calcium and magnesium present in the original paper, or

(2) double the total number of μg ions of calcium and magnesium present.

Both of these relationships can be interpreted in terms of an ion exchange process.

The adsorption of hydrogen ions through the displacement of sodium, potassium, calcium and magnesium ions requires the assumption that the alkaline earths (Ca and Mg) are held on the paper in the form of a basic salt or a mixed salt. The existence of such basic or mixed salts has been predicted by CHURCH[3] from his studies on cellulosic insulating materials.

On the other hand, the adsorption of hydrogen ions by exchange with calcium and magnesium ions only requires the assumption that sodium and potassium ions are not held by the paper on exchange sites. This assumption is supported by the fact that the alkalis were completely liberated by washing the paper with water while the alkaline earths were only partially removed by water (*cf.* Table II). Similar observations were made by WALKER AND QUELL[4] when they analysed the ash of cotton which had been washed with water, acids or salt solutions.

The exchange relationships observed with the filter papers were not maintained with the regenerated fibres. This can be attributed to displacement of the alkaline earth metal ions and the occlusion of alkaline salts during the manufacturing process.

Ion exchange by cotton fibres has been attributed to acid groups on the fibre[4]. In natural fibres, the exchange capacity was found to be principally accounted for by the materials associated with the cellulose such as pectic substances, lignin etc.,[5,6] while in fibres freed of these substances, the exchange capacity was attributed to acid groups on the cellulose itself[5,7–9].

Convincing evidence for exchange through acid groups on cotton is given by SOOKNE AND HARRIS[11] who determined the base-combining capacity of cotton by treating acid-washed and electro-dialysed cotton with salt solutions. Complete saturation was only obtained using a salt of a very weak acid. Esterification of the acid groups with diazomethane reduced the base-binding capacity to nearly zero.

The suggestion that groups other than hydroxyl groups are responsible for the acid nature of cellulose is supported by the fact that the ash content and adsorptive capacities of Whatman No. 1 filter paper and cellulose acetate fibre were found to be similar (Table III) although two of the hydroxyls of the normal cellulose structure exist as acetyl groups in the acetate fibre. Viscose rayon is reported[12] to contain a higher proportion of carboxyl groups than pure cotton and this property is reflected in Table III by the higher ash content and greater adsorptive capacity for hydrogen ions.

The results quoted in this study could be attributed equally well to the removal of impurities by simple dissolution rather than by an ion exchange process. The latter interpretation is preferred, however, since it is not only in agreement with the conclusions of many previous studies on cellulose products but it also provides an explanation for the adsorption of metal ions.

SUMMARY

The adsorption of hydrogen ions by filter paper may be attributed to a process of ion exchange and the exchange capacity of Whatman No. 1 filter paper was found to be $1.08 \cdot 10^{-5}$ g per g of dry paper. This value is similar to the value reported[5] for dewaxed, depectinised cotton ($1 \cdot 10^{-5}$ g per g of dry cotton).

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INORGANIC ADSORPTION PAPER CHROMATOGRAPHY

III. THE ADSORPTION OF DIVALENT METAL IONS BY FILTER PAPER

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(Received April 25th, 1960)

The adsorption of copper, lead, cadmium, zinc and nickel ions by Whatman No. 1 filter paper has been examined as part of an investigation of the possible influence of adsorption in inorganic paper chromatography. An earlier chromatographic study[1] indicated that these divalent ions are less strongly held than the hydrogen ions previously examined[2].

PROCEDURE

One-grams samples of filter paper were allowed to come to equilibrium with 20 ml of dilute metal nitrate solution. The equilibrium concentration of metal ion was accurately estimated and by comparison with the original solution, the number of moles of cation adsorbed per gram of dry paper was calculated from the observed change in concentration. The concentration of metal nitrate solution in contact with the paper was varied and tests were made at time intervals to assess the rate of adsorption. Other electrolytes were included in some solutions and their influence on the amount of adsorption was observed.

Several analytical techniques were applied in this investigation. In one series of experiments, aliquots of the solutions were titrated in ammoniacal buffer solution with a standard solution of disodium ethylenediaminetetraacetate. This procedure gave the total amount of metal ion present in the solution together with any calcium and magnesium ions released from the paper.

The studies were then repeated using techniques which were specific for the metal ions. Copper and nickel solutions were analysed colorimetrically (as the copper tetrammine and oxidised nickel dimethyl glyoxime complexes respectively) with the aid of a Unicam S.P.500 Spectrophotometer. Lead, cadmium and zinc solutions were analysed polarographically using a Tinsley Recording Polarograph.

Adsorption of the five cations was found to occur rapidly. The amount of cation adsorbed by Whatman No. 1 filter paper from solutions of different concentrations is recorded in graphical form on Fig. 1. These plots of the logarithm of the number of gram ions of metal adsorbed per gram of paper against the logarithm of the equilibrium concentration of solute indicate a simple linear relationship. Similar plots of the amount adsorbed against the initial concentration of solute were also linear.

This suggests that the relationship between the uptake of cations and concentration could be expressed in terms of either the Freundlich or Boedeker equations. Both these equations have been found to be applicable to ion exchange equilibria[3].

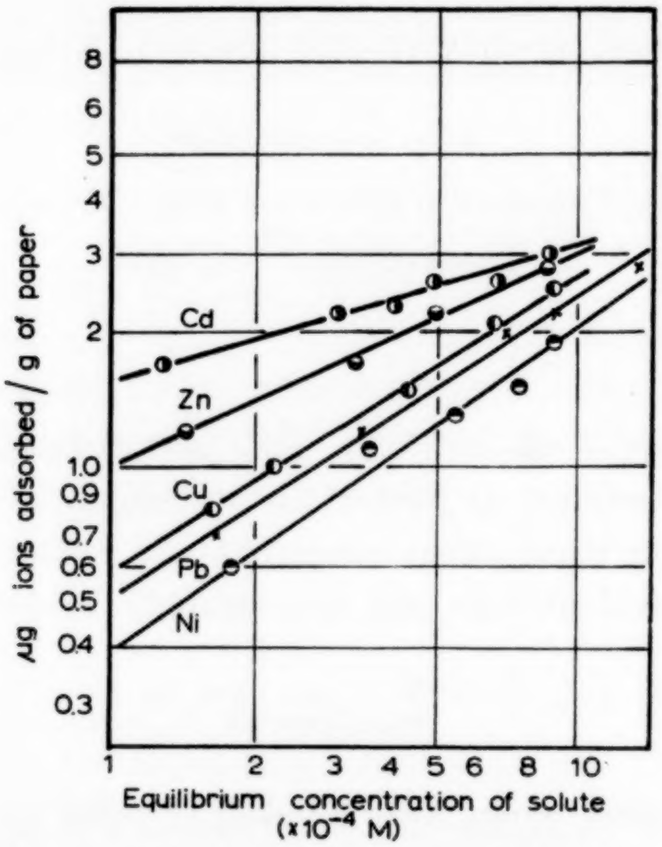

Fig. 1. Graphs illustrating the logarithmic relationship existing between the amount of metal ion adsorbed and the concentration of metal ion in the solution.

By using more concentrated solute solutions ($2.5 \cdot 10^{-3}$ to $10^{-2}\,M$) a constant adsorption value was obtained indicating saturation of the paper in respect to each ion.

The substitution of values from Fig. 1 in the Langmuir adsorption isotherm permitted the calculation of the saturation value of the paper for a particular solute. The calculated and experimental values obtained for the adsorptive capacity of Whatman No. 1 filter paper are recorded in Table I.

The titrations with standard disodium ethylenediaminetetraacetate solution indicated that the total concentration of metal ions in the solution did not change when the metal nitrate solutions were brought in contact with the filter paper. Since the adsorption of copper, lead, zinc, cadmium and nickel ions was conclusively

TABLE I

THE ADSORPTIVE CAPACITY OF WHATMAN NO. 1 FILTER PAPER FOR DIVALENT METAL IONS

Metal ion	*Microgram ions adsorbed per g of paper*	
	Experimental value	*Calculated value*
Cu	3.9	4.1
Pb	—	4.0
Zn	4.1	3.8
Cd	3.6	3.5
Ni	3.3	3.6

demonstrated by the results recorded in Fig. 1 and Table I, the titration results can only be explained by postulating the liberation of a calcium or magnesium ion from the paper for every other metal ion adsorbed. This postulate was confirmed with cadmium and zinc by adding potassium cyanide to the solution prior to titration. In this manner, the calcium and magnesium could be titrated while the other metal ions remained as the cyanide complexes.

The results shown in Table II illustrate how the adsorption of the divalent metal ions was greatly reduced in the presence of competing electrolytes. The presence of an equivalent amount of potassium or magnesium nitrates had little effect on the amount adsorbed, but the presence of higher concentrations markedly reduced the uptake of metal ions by the paper. The divalent ion was a more efficient suppressor than the monovalent ion as would be expected for an ion exchange reaction. The addition of an equivalent amount of acid greatly reduced but did not eliminate adsorption of the cations. Higher concentrations of acid or the presence of another competing salt did reduce adsorption to zero. Results in the presence of potassium biphthalate buffer (pH 4) are difficult to interpret since complex formation may be responsible for the non-adsorption of copper, nickel and cadmium ions.

TABLE II

THE EFFECT OF ELECTROLYTES ON THE AMOUNT OF CATION ADSORBED BY WHATMAN NO. I FILTER PAPER

Electrolyte solution 0.001 M metal nitrate in	*Microgram ions adsorbed per g of paper*				
	Cu^{2+}	Pb^{2+}	Ni^{2+}	Cd^{2+}	Zn^{2+}
Distilled water	2.4	2.2	2.2	3.0	2.6
0.001 M KNO_3	1.9	2.4	2.1	3.0	2.2
0.010 M KNO_3	1.1	1.4	1.3	2.9	1.5
0.100 M KNO_3	0.9	0.6	0.7	1.2	0.8
0.001 M $Mg(NO_3)_2$	2.0	2.3	2.0	2.8	2.1
0.010 M $Mg(NO_3)_2$	0.6	1.0	1.4	1.9	0.8
0 100 M $Mg(NO_3)_2$	0.4	0.4	0.5	1.0	0.7
0.001 M HNO_3	0.2	1.4	0.6	0.4	n.d.
0.001 M HNO_3+ 0.1 M KNO_3	n.d.	0.4	0.1	n.d.	0.2
0.010 M HNO_3	n.d.	n.d.	n.d.	n.d.	n.d.
0.010 M HNO_3+ 0.1 M KNO_3	n.d.	n.d.	n.d.	n.d.	n.d.
0.100 M HNO_3	n.d.	n.d.	n.d.	n.d.	n.d.
0.05 M K biphthalate	n.d.	—	n.d.	n.d.	0.8

n.d. = none detected.

DISCUSSION

The low adsorptive capacity of the filter paper introduced a number of factors which limited the general accuracy of the quantitative determinations to an estimated ±10%. Despite this limitation, the evidence for adsorption of the divalent metal ions through a process of ion exchange is fairly conclusive.

The titration with disodium ethylenediaminetetraacetate solution showed that a calcium or magnesium ion was displaced into solution for every divalent metal ion adsorbed by the paper. The amount of divalent metal ion adsorbed was reduced by the presence of competing electrolytes in the solution. The efficiency of hydrogen ions in reducing adsorption can be taken to indicate exchange through weak acid groupings. Finally, the adsorption data were consistent with theoretical equations that have been applied to ion exchange reactions.

On the other hand, the estimated adsorptive capacity of the paper for the bivalent metals (approximately $4 \cdot 10^{-6}$ g ions per g of paper) was less than the total amount of calcium and magnesium ions liberated from the paper by acid treatment ($5.6 \cdot 10^{-6}$ g ions per g of paper) although it is similar to the amount of alkaline earth retained by the paper when placed in distilled water ($4.1 \cdot 10^{-6}$ g ions per g of paper).

The incomplete replacement of the alkaline earths on the paper by the copper, lead, cadmium zinc and nickel ions may be attributed to a similarity in the exchanging power of all divalent cations with preferential retention of the calcium and magnesium ions originally present on the exchanger.

The adsorption of polyvalent ions by cellulose has received little attention. GAVORET[4] studied the adsorption of lead, thorium and copper salts by purified cotton; IVANOVA and co-workers[5] studied the adsorption of nickel and ferric ions by oxidised cellulose; and SCHÖNFELD AND BRODA[6] examined the adsorption of lead ions by lignin-free paper. Each of these workers has suggested that the mode of adsorption is ion exchange through carboxyl groups.

SUMMARY

The adsorption of divalent metal ions by Whatman No. 1 filter paper may be attributed to a process of ion exchange. The exchange capacity for the divalent metal ions was found to be approximately $4 \cdot 10^{-6}$ g ions per g of paper.

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INORGANIC ADSORPTION PAPER CHROMATOGRAPHY

IV. THE ADSORPTION OF FERRIC IONS BY FILTER PAPER

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Earlier papers in this series have described a chromatographic study of the adsorption of cations by filter paper[1] and quantitative studies on the adsorption of hydrogen ions[2] and divalent metal ions[3]. The chromatographic study indicated that trivalent metal ions were strongly retained by the paper support. Quantitative data on the adsorption of trivalent cations has now been obtained by examining the adsorption of ferric ions.

PROCEDURE

One-gram samples of filter paper were allowed to stand covered with dilute, freshly prepared ferric nitrate solutions. The concentration of ferric ion remaining in the solution was determined colorimetrically at different time intervals. (Aliquots of the solution were acidified and thiocyanate ions added. The intensity of the red ferric thiocyanate complex was measured in a Unicam S.P.500 spectrophotometer.) The changes in ferric ion concentration were used to determine the number of gram ions of ferric iron adsorbed per gram of paper. The effect of electrolytes on the amount adsorbed was studied by preparing solutions of fixed ferric ion concentration containing varying concentrations of other electrolytes. These results are recorded in Table I.

In contrast to the negligible effect of time on the amount of divalent or hydrogen ion adsorbed, the adsorption of ferric ions increased markedly with time. This effect was confirmed by studying seven different grades of filter paper and the results obtained are summarized in Fig. 1. It was observed that although the actual amount adsorbed from a solution of a given concentration increased with increasing ash content of the paper, the effect of time was similar in all cases.

With longer periods of contact between the paper and ferric nitrate solutions, the solutions darkened markedly in colour indicating that the ferric ions were being rapidly hydrolysed. According to POKRAS[4] the products of hydrolysis include hydroxy compounds of lower positive charge (*e.g.* $[Fe(OH)]^{+2}$, $[Fe(OH)_2]^{+}$) and stable dimeric compounds (*e.g.* $[Fe_2(OH)_2]^{4+}$).

The increased adsorption with time of standing (as shown in Fig. 1) can therefore be attributed to either the ability of the paper to retain a larger number of ions of lower charge or the removal of ferric ions from the solution by irreversible hydrolytic reactions, *e.g.* dimerisation. The effects of hydrolytic reactions were minimized by acidifying the test aliquots prior to estimating the iron content and by comparing the test solutions with blanks retained in similar glass vessels for similar periods of time. The increase in adsorption with time of standing therefore appears to be related to the adsorption of the hydroxy compounds. This idea is supported by the fact that the papers became distinctly yellow in colour after prolonged contact with the solutions. The adsorption of heavy and hydrolysable ions as metal hydroxides was also suggested by GAVORET[5] in her study of the adsorption of lead and thorium salts by purified cotton.

TABLE I

THE EFFECT OF ELECTROLYTES ON THE ADSORPTION OF FERRIC IONS BY WHATMAN NO. 1 FILTER PAPER

Electrolyte solution 10^{-3} M ferric nitrate in	*pH*	*Moles Fe^{3+} adsorbed per g dry paper ($\times 10^6$)*
Distilled water	3.5	5.6
10^{-3} *M* KNO_3	3.3	5.4
10^{-2} *M* KNO_3	3.3	5.3
10^{-1} *M* KNO_3	3.4	5.0
10^{-3} *M* $Mg(NO_3)_2$	3.5	5.6
10^{-2} *M* $Mg(NO_3)_2$	3.5	5.5
10^{-1} *M* $Mg(NO_3)_2$	3.3	4.6
10^{-3} *M* HNO_3	3.2	3.2
10^{-3} *M* HNO_3 + 0.1 *M* KNO_3	3.2	3.3
10^{-2} *M* HNO_3	2.1	0.9
10^{-2} *M* HNO_3 + 0.1 *M* KNO_3	2.2	0.9
10^{-1} *M* HNO_3	1.3	none detected
0.05 *M* K biphthalate	4.2	1.0

Time of contact between paper and solutions, 2 hours.

The detailed study using Whatman No. 1 filter paper showed that the amount adsorbed from freshly prepared solutions did not vary much in the first 15 minutes. The effect of solute concentration on the amount adsorbed during this time period is illustrated by the logarithmic plots drawn on Fig. 2. The points approximate a straight line indicating agreement with the Freundlich equation. An experimental adsorptive capacity of $3.2 \cdot 10^{-6}$ g ions per g of paper was obtained for periods of contact of less than 15 min.

This is approximately one third of the estimated adsorptive capacity of the paper for hydrogen ions[2].

The results recorded in Table I illustrate the influence of salts and hydrogen ions on the adsorption of ferric ions. The effect of electrolytes in the solution on the amount adsorbed was not so pronounced as with the divalent metals. This suggests that the bond between the paper and the ferric ion is stronger than the bond formed with divalent ions.

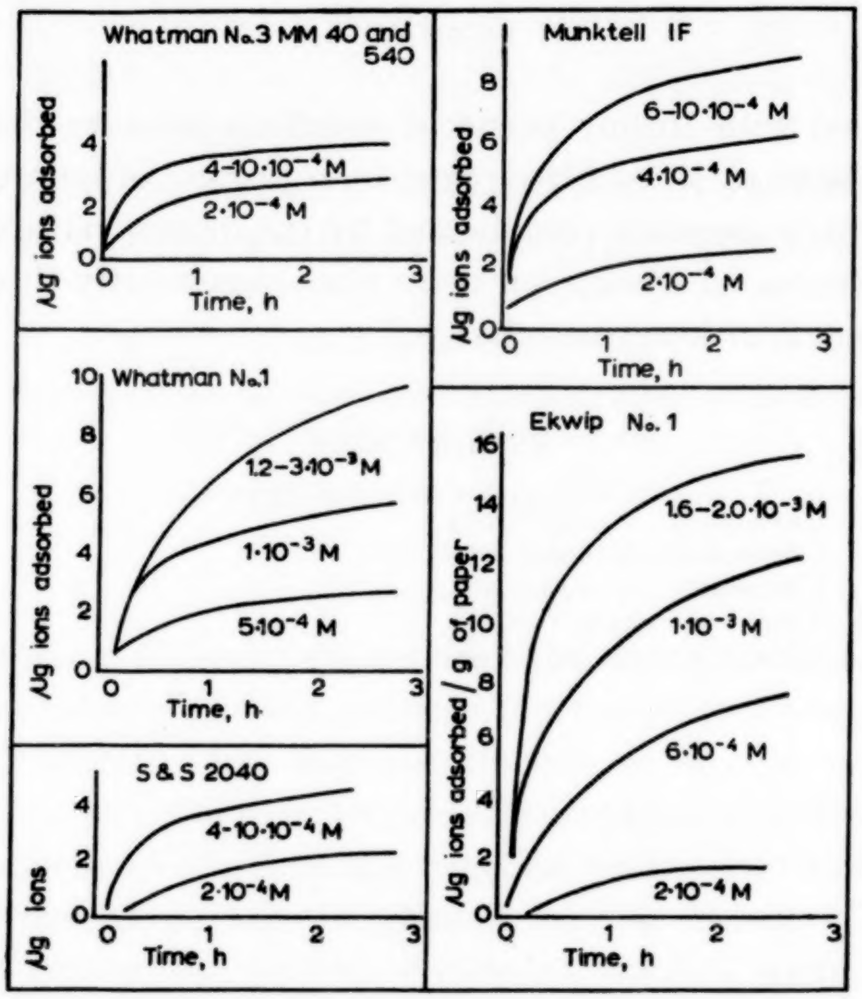

Fig. 1. Graphs showing the effect of time of standing and grade of paper on the adsorption of ferric ions by filter paper.

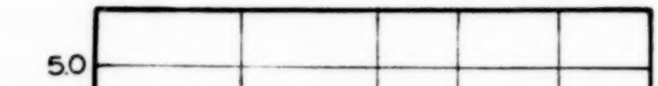

Fig. 2. Graph illustrating the logarithmic relationship existing between the amount of ferric ion adsorbed by Whatman No. 1 filter paper in the first 15 min of contact and the concentration of ferric ions in solution.

SUMMARY

The results obtained with freshly prepared solutions indicate that ferric ions may be adsorbed by Whatman No. 1 filter paper by a process of ion exchange. However, on standing the system becomes complicated by the diverse nature of the hydrolysis products formed. Increased adsorption takes place, apparently through the retention of hydroxy compounds of lower net charge.

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CHROMATOGRAPHIE VON HYDROXYLIERTEN FLAVANDERIVATEN AN CELLULOSEGLYKOLSÄUREÄTHER

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Bei der Darstellung reiner Flavone bereitet die Abtrennung von störenden Substanzen nicht nur erhebliche Schwierigkeiten, sondern führt auch zu Verlusten.

Die hartnäckig anhaftenden Begleitstoffe können sich so störend bemerkbar machen, dass sie trotz wiederholtem Umkristallisieren die Kristallbildung verzögern und in pharmakologischen Versuchen das Ergebnis verfälschen können.

Die allgemein bekannten Schwierigkeiten, wie sie bei der Darstellung reiner Flavone auftreten, können weitgehend vermieden werden, wenn die Auszüge aus den Pflanzen vor der weiteren Aufarbeitung einer Reinigung unterworfen werden. Dabei sollten zweckmässig solche Massnahmen nicht zur Anwendung gelangen, die in einer Behandlung der Auszüge mit Chemikalien bestehen, wie z.B. einer Bleisalzfällung. Der früher übliche Gebrauch bei den Aufarbeitungen z.B. Bleisalze zu verwenden war nicht zu umgehen, weil geeignete andere Verfahren noch nicht bekannt waren. Auch heute werden noch häufig Bleisalze verwendet, obgleich bessere Verfahren zur Verfügung stehen. Durch Adsorptionsmittel lassen sich einige die Darstellung reiner Flavone störender Substanzen ausschalten.

Die Anwendung von z.B. Aluminiumoxyd, ist auf ganz bestimmte Flavanderivate beschränkt. Ein anderes Adsorptionsmittel ist das von WENDER[1] vorgeschlagene Magnesium-2.5-silikat. WENDER, MORRIS UND GAGE[2] haben den Austaucher IRC-50 (H-Form) zur Isolierung von Flavonen aus wässrigen Pflanzenextrakten empfohlen.

Einen wesentlichen Fortschritt, die Darstellung reiner Flavone erleichtert zu haben, verdanken wir HOERHAMMER UND WAGNER[3], die das von GRASSMANN und Mitarb.[4] verwendete Polyamid als Adsorptionsmittel erstmalig auf dem Flavongebiet systematisch prüften. Der Einfluss des Polyamids auf Flavonoide ist eine ziemlich feste Bindung der Flavon-Aglykone, während Flavonglykoside praktisch nicht festgehalten werden. Die Aglykone werden reversibel adsorbiert. Der Vorteil des Polyamids ist eine Entfernung der Begleitstoffe, die hauptsächlich die Darstellung reiner Flavone erschweren. Die Adsorption ist eine irreversible solcher Flavanderivate, wie der Leucocyane und Catechine, die für eine phytochemische Untersuchung jedoch nicht bedeutungslos sind. Die Entfernung der Gerbstoffe durch Polyamid kann dagegen für die weitere Aufarbeitung vorteilhaft sein. Die Vorteile der Polyamidadsorption haben wir in vielen Fällen für die Darstellung der Flavone ausnützen können.

Für phytochemische Arbeiten ist es wichtig, ein solches Adsorptionsmittel zu verwenden, das noch selektiver eine Abtrennung störender Begleitstoffe ermöglicht, aber alle wichtigen Inhaltsstoffe in derart gereinigten Auszügen wiederzufinden ermöglicht. Mit einer Entfernung der praktisch nicht zu beeinflussenden, stets bei der Extraktion von Pflanzen in den Auszug gelangenden, oder zich beim Erhitzen bildenden Polymerisate müsste sich auch die weitere Aufarbeitung erleichtern lassen.

Wir kamen während unserer Arbeiten über die Inhaltsstoffe des Weissdorns dazu, das neu gefundene und an der Wirkung wesentlich beteiligte *l*-Epicatechin[5] nicht durch eine praktisch irreversible Adsorption zu verlieren.

Auf Grund der von LETZIG[6] beschriebenen Untersuchungen, zeigen Gerbstoffe auf Celluloseäther eine ausflockende Wirkung, ebenso verhält sich *d*-Catechin. Wie wir feststellen, verhält sich dagegen *l*-Epicatechin als Isomeres vom *d*-Catechin nicht in gleicher Weise gegenüber Celluloseäthern. In Anbetracht der Wasserlöslichkeit von Celluloseäthern ist deren Verwendung als Adsorptionsmittel nicht ohne weiteres möglich, wenn sie nicht durch weitere chemische Umsetzung bei Erhaltung ihrer Eigenschaften modifiziert werden. Das legte den Gedanken nahe, zunächst die leicht zugänglichen Celluloseglykolsäureäther zu prüfen.

Bei einem orientierenden Versuch einen wässrigen Auszug aus Akazienblüten zu reinigen, erfolgte eine so weitgehende selektive Entfernung von Ballaststoffen, dass aus dem eingedampften Durchlauf, trotz der vorhandenen grossen Zuckermenge, die gallertartige Ausscheidung der Acacetinglykoside eintrat.

Für die vorliegenden Arbeiten wurde als Adsorptionsmittel die modifizierte Cellulose CAM (Schleicher und Schüll) verwendet. Die Ergebnisse sollen an Hand der eingesetzten Ausgangsmaterialien beschrieben werden. Als ein wesentliches Ergebnis aus den bisherigen Versuchen ist zunächst festzustellen, dass die Celluloseglykolsäureäther die in fast allen Pflanzenauszügen vorhandenen oder sich im Verlauf der Aufarbeitungen bildenden polymeren Anteile irreversibel adsorbieren. Gegenüber dem Polyamid besteht der grundlegende Unterschied, dass die Flavane, (+)-Catechin und (—)-Epicatechin, nicht durch die Celluloseglykolsäureäther dem Auszug entzogen werden.

Das angegebene Verhalten gibt die Möglichkeiten, die bekannten Inhaltsstoffe in Pflanzenauszügen vollständiger zu erfassen, als das mit Polyamid in gleicher Weise möglich ist, bzw. durch Kombination der beiden Adsorbentien eine weitere Selektion zu erreichen. Das ist praktisch von Bedeutung, um die Flavonoide, Leucocyanidine und Catechine von den Polymerisationsprodukten abzutrennen.

Die Pflanzenauszüge erfahren eine deutliche Aufhellung, wobei das Adsorbens in feuchtem Zustand eine starke Verfärbung erkennen lässt. Eine Extraktion des Adsorbens mit Methanol entfernt nicht mehr die polymeren Anteile.

Das im Handel befindliche Fisetin und Robinetin ist sehr stark verunreinigt. Nach einem Durchgang über eine Celluloseglykolsäureäther-Säule sind die unerwünschten Nebenbestandteile soweit entfernt, dass ein rein gelbes Fisetin ausfällt. In den Fisetholz-Extrakten befinden sich Fisetin und Dihydrofisetin neben Ballaststoffen. Die Reinigung über eine Cellulose-Säule haben FREUDENBERG UND ROUX[7] beschrieben. Die Arbeitsweise ist dann befriedigend, wenn die in dem rohen Auszug ebenfalls vorhandenen und nicht geringen Mengen anderer Extraktstoffe vorher bereits abgetrennt sind. GERNGROSS UND HÜBNER[8], die für ihre Untersuchung aus Fisetholzextrakt das Fisetin isolierten, beschreiben die weitere Reinigung des bereits vorgereinigten Produktes durch wiederholtes Umfällen. Dagegen führt eine Adsorption der Verunreinigungen an CAM-Cellulose mühelos zu einer sauberen Substanz.

Der Vorteil der CAM-Cellulose tritt besonders charakteristisch hervor, wenn pflanzliche Arzneimittel untersucht werden sollen. Als wesentliche Voraussetzung wurde eine praktisch vollständige vorherige Entfernung des Extraktionsmittels für erforderlich gefunden. Die Abtrennung von die Analyse störenden Begleitstoffen mit Bleiacetat ist mit CAM-Cellulose vorteilhafter. Die Flavane, *d*-Catechin und *l*-Epicatechin, werden nicht festgehalten, sondern im Durchlauf gefunden. Das lässt sich am einfachen Beispiel bei der Verwendung der beiden Catechine zeigen. Die Säule hält braunrote Produkte zurück und die beiden Substanzen befinden sich im Durchlauf. Ein noch besseres Beispiel ist die Catechu-Urtinktur. In 2%iger Essigsäure wird papierchromatographisch die Tinktur über die ganze Laufstrecke braungefärbt verzogen und gibt einen tiefbraunen Startpunkt. Nach Adsorption an CAM wird der wässrige Durchlauf mit Butanon extrahiert, bzw. die Säule mit Methanol gewaschen. In 2%iger Essigsäure zeigt der wässrige Durchlauf nur einen rein gelbgefärbten Startpunkt, ebenso der Butanon-Auszug bei gleichzeitig gelber Laufstrecke bis zur Hälfte, die das Ausgangsmaterial zeigt. Der Methanoldurchlauf weist nur einen schwach gelben Startpunkt auf. Die Farbreaktionen werden mit dem stabilen Diazoniumsalz Echtschwarzsalz K^{R}[9] erhalten. In Partridge-Gemisch und *p*-Dimethylaminozimtaldehyd als Reagenz zeigt das Ausgangsmaterial einen blaugefärbten Startpunkt, der Butanonextrakt durch Blaufärbung die beiden Catechine an.

Die selektive Adsorption der CAM zeigt ein Vergleich mit Cellulosepulver (Whatman I). Das Papierchromatogramm der Fraktionen aus Catechu in 2%iger Essigsäure verhält sich wie die Ausgangssubstanz.

Handelsübliche Rutin gibt nach der Hydrolyse mit 1%iger Schwefelsäure unter Druck im oberen Rand der Säule eine braungefärbte Zone. Die Eluate mit Wasser und Methanol werden getrennt eingedampft. Aus dem Methanol-Rückstand (47.7%, Smp. 275–280°) nach erneuter Chromatographie und Eluieren mit Wasser und dann mit reinem Methanol, fielen gelbe Kristalle an, Smp. 303-304°.

Aus handelsüblichem Quercitrin, gelöst in 30%igem Methanol, konnte mit CAM eine diese stark braun färbende Substanz entfernt werden. Der Smp. für das Ausgangsmaterial betrug 172–174°, der nach der Adsorption auf 182–184° (Lit. 182–184°) anstieg.

Aus dem marktgängig gelblich gefärbten Hesperidin, das auf der CAM-Säule nacheinander mit 30 und 50%igem Methanol gewaschen wurde, fielen mit reinem Methanol schneeweisse Kristalle mit dem Smp. 253–255° (Lit. 257–261°) an.

Naringin wurde mit Acetanhydrid acetyliert, der Überschuss mit soviel in Wasser aufgeschlemmter CAM hydrolysiert, das Essigsäure gebildet wird, auf eine kurze Säule gegeben und mit Wasser bzw. mit 30%igem Methanol gewaschen. Aus dem ersten Durchlauf fielen 165.6 mg (Smp. 116–120°) und mit 30%igem Methanol 2.5 mg (Smp. 119–120°) an.

Rohes Dihydroquercetin wurde mit CAM fein verrieben, auf eine ebensolche Säule gegeben und mit 20 und 50%igem Aceton eluiert. Aus dem 20%igen Aceton wurde papierchromatographisch einheitliches Dihydroquercetin erhalten, wie der Nachweis mit Rhodamin B[10] zeigte. Das 50%ige Aceton erhielt reines Quercetin. Die Startpunkte waren rein weiss. Das Ausgangsmaterial zeigte in Partridge-Gemisch im U.V.-Licht eine die halbe Laufstrecke einnehemende Verunreinigung und mit Rhodamin B färbte sich die gesamte Laufstrecke an. Der Vorteil der Trennung über eine CAM-Säule zeigt sich gegenüber der sonst üblichen Reinigung von Dihydroflavonolen. So waren z.B. für die Darstellung eines reinen Dihydrokämpferols 10 Kristallisationen aus absolutem Äthanol notwendig[11].

Ein Vergleich mit Cellulosepulver färbte den mit 20%igem Aceton anfallenden Durchlauf im PCG mit Rhodamin B über die ganze Laufstrecke an. Der 20 und der 50%ige Acetondurchlauf gab nach dem PCG am Startpunkt mit Rhodamin B und mit Diphenylborinsäure[12] eine Reaktion.

Die von NEU[13] an Polyamid beschriebene Trennung von Chalconen und Flavanonen ist in gleicher Weise auch an Celluloseglykolsäureäther möglich. Aus einem Isomerisierungsgemisch mit 2',4-Dihydroxychalcon und 4'-Hydroxyflavanon konnten auf einer CAM-Säule mit 30%igem Methanol 66% Flavanon mit dem Smp. 112–114° abgetrennt werden. Mit reinem Methanol wurde das Chalcon eluiert. Sowohl die offene als auch die geschlossene Form zeigte im PCG, dass eine scharfe Trennung erfolgt war. Im Vergleich zum Polyamid ist die von CAM-Cellulose adsorbierte Menge wesentlich geringer.

DANK

Verfasser dankt der Firma Carl Schleicher u. Schuell, Dassel/Kr. Einbeck für die zur Verfügung gestellte CAM-Cellulose.

ZUSAMMENFASSUNG

Die Verwendung von Celluloseglykolsäureäther zur Chromatographie für die Gewinnung von Flavonen, Flavanonen und Flavanolen und den ihnen entsprechenden Glykosiden wird in Beispielen gezeigt.

Celluloseglykolsäureäther adsorbiert aus Pflanzenauszügen irreversibel die polymeren Anteile.

Handelsübliche Flavonol- und Flavanglykoside können mit Celluloseglykolsäureäther zu reinen Substanzen aufgearbeitet werden.

Die Flavane, *d*-Catechin und *l*-Epicatechin, werden an Celluloseglykolsäureäther nicht, wie bei der Verwendung von Polyamid, irreversibel adsorbiert.

Die Trennung von Flavonolen und ihren Dihydroderivaten gelingt an Celluloseglykolsäureäther-Säulen ebenso wie an Polyamid.

Die Trennung von Chalconen und Flavanonen ist mit Celluloseglykolsäureäther als Adsorbens ebenso wie auf Polyamid möglich.

Im Vergleich zu Polyamid ist das Aufnahmevermögen von Celluloseglykolsäureäther geringer.

SUMMARY

The use of carboxymethylcellulose as adsorbent in chromatography for the preparation of flavones, flavanones and flavanols and their glycosides, is described.

Carboxymethylcellulose irreversibly adsorbs the polymeric components of plant extracts.

Commercial flavonol and flavan glycosides can be purified by means of carboxymethylcellulose.

The adsorption of the flavans *d*-catechin and *l*-epicatechin on carboxymethylcellulose is not irreversible, in contrast to that on polyamide.

The flavonols and their dihydro-derivatives can be separated on carboxymethylcellulose columns as well as on polyamide.

Chalcones and flavanones can be separated with carboxymethylcellulose as adsorbent, as well as with polyamide.

The adsorptive capacity of carboxymethylcellulose is less than that of polyamide.

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Short Communications

The use of ion exchange resin loaded paper for "desalting" small samples prior to paper chromatography*

The separation of non-ionic compounds by paper chromatography is often seriously impaired by the presence of salts in the samples to be chromatogrammed. Interfering salts are most conveniently removed by passage of the sample through a mixed bed ion exchange resin column, or by the direct addition of resin to the sample. These procedures usually dilute the sample. For small samples, an alternative process utilizing ion exchange resin loaded paper is described below. The sample is eluted successively from papers loaded with cation and anion exchange resin, respectively, directly onto the origin on a sheet of chromatography paper. Using this procedure, an amount of material no larger than the actual sample to be chromatogrammed may be treated to remove ionic substances. The method has been used for the separation on paper of phosphorylated carbohydrate intermediates of photosynthesis and respiration after treatment with phosphatase. Relatively large amounts of buffer and other ionic compounds were present.

The sample to be chromatogrammed was applied 1 cm from one end of a 1.5 cm × 6 cm strip of ion exchange paper in the H^+ form (ion exchange resin loaded papers containing Amberlite resin IR-120, IRC-50, IRA-400, or IR-4B are manufactured by H. Reeve Angel and Co., Inc., Clifton, New Jersey). A 1.5 cm × 6 cm strip of ion exchange paper in the OH^- form was attached to the other end (Fig. 1). Water was then allowed to descend the ion exchange papers. Water-soluble non-ionic compounds ran with the solvent front and were eluted onto the origin of a sheet of chromatography paper[1]. It was important that the ion exchange papers were thoroughly washed before use, otherwise colored impurities from the papers were eluted. The size of the spot at the origin was controlled by the rate of airflow directed onto the underside of the chromatography paper. To maintain a humid atmosphere, part of the inside of the covering glass tank was lined with absorbent paper saturated with water.

The water for elution was applied to the ion exchange paper from a container similar to that described by GUNEW[2], except that the container was made of parafilm instead of thin polyethylene tubing. The parafilm was substituted since it had more rigidity and was easier to seal. It also was found convenient to use a small piece of parafilm to join the ion exchange paper strips together (Fig. 1). The use of a parafilm container for supplying the eluting water, rather than techniques employed in conventional descending chromatography or elution such as hanging the upper end of the paper strip over the edge of a water trough, was preferred for the following reasons: (1) it allowed the use of a simple supporting stand (Fig. 1) so that the height and position of the strip could be conveniently adjusted, (2) it allowed a precise control of the volume of eluant used. For ion exchange paper strips of the dimensions described in Fig. 1, 0.45 ml of water was required to saturate the strips. Hence, the volume of eluate was predetermined by the volume of water added to the parafilm container. After the addition of the water, the air was turned on so that the elution system required no further attention until after the predetermined volume of eluate had been transferred to the chromatography paper. The total elution time for the transfer of 50–100 μl of eluate to the paper was approx. 45 min.

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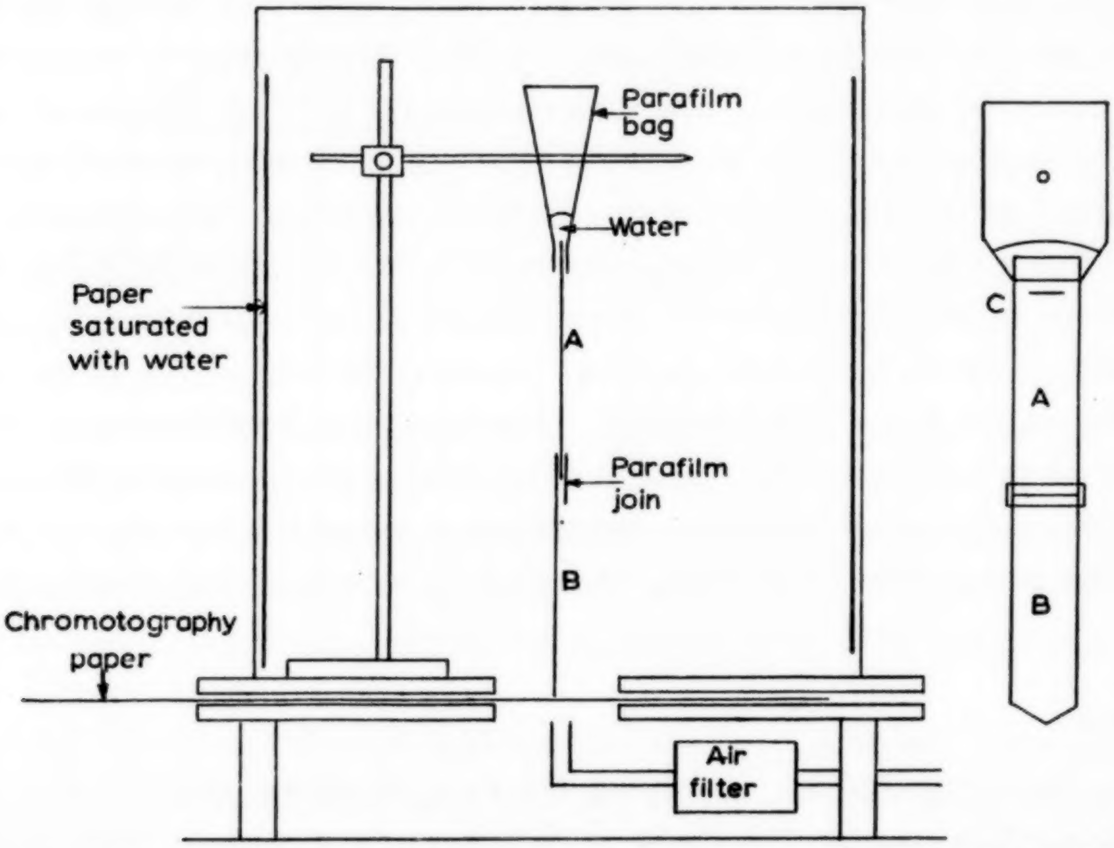

Fig. 1. Elution system. A = ion exchange paper, grade WA-2 (loaded with Amberlite IRC-50, 4.6–5.0 mequiv. per dry g of paper); B = ion exchange paper, grade WB-2 (loaded with Amberlite IR-4B, 4.0–4.2 mequiv. per dry g of paper); C = point of application of sample.

The procedure as described above may also be applied in instances where it is advantageous to convert small samples to a different ionic form before chromatography. This may be conveniently accomplished by substituting in the elution system a single strip of ion exchange paper in the required ionic form.

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The use of silicone rubber gums or grease in low concentration as stationary phase for the high temperature gas chromatographic separation of lipids*

In spite of the fact that silicone oils, greases and gums have had widespread use in gas chromatography and have been used at high temperatures[1-7], very few references have been made to their use at low concentrations (5 % of substrate) and at high temperatures (240 to 300°). It was found that such columns showed unusually fast retention times while still maintaining excellent resolution, thus making them very useful for the analysis of many lipid substances or their thermally stable derivatives. It is true that decreasing the level of the liquid phase also decreases the capacity of the column, so that for many analyses where collection of fractions is a primary concern this would be a disadvantage. However, the inconvenience in having to collect more often is far offset by the real advantages to be gained by shorter retention times, namely, more rapid analyses, shorter exposure of the sample to high temperatures and the possibility of analysis of many substances that could otherwise not be done.

Experimental

The packing was prepared by dissolving the gum or grease in enough chloroform to just wet the inert suspending medium (firebrick, 42–60 mesh, or Chromosorb W80–100 mesh can be used), then removing the solvent under vacuum on a rotary evaporator. The columns were made of 1/4 in. stainless steel tubing (4 mm i.d.) and were packed by tapping the column on the floor while adding the packing.

Two silicone rubber gums (Union Carbide's KS-1014 and W-96) were tried as well as Dow Corning's high vacuum silicone grease. All gave essentially the same results at 250°. The inert ingredients in KS-1014, which are silica aerogel and $CaCO_3$, apparently did not affect separations.

The chromatography was carried out on an Aerograph (Wilken's Instrument Company) using a Brown Honeywell recorder (1 mV full scale deflection) equipped with a Disc Integrator.

Helium was the carrier gas. Samples were injected with a 10 μl or 100 μl Hamilton syringe. Sample sizes of 1 μl of individual hydrocarbons could be injected without overloading.

Results

Table I shows the change in retention time for 5 foot columns in which the proportion of silicone rubber varies from 5 % to 25 % of the weight of the firebrick. It can be seen that the retention times at 5 % liquid phase are about one-third those at 25 % and this occurs at no loss in resolution.

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TABLE I

CHANGE IN RETENTION TIME WITH VARYING LEVELS OF LIQUID PHASE

Parts liquid phase per 100 parts firebrick	*Retention times (min)*		
	5%	*15%*	*25%*
Methyl myristate	0.3	0.8	1.3
Methyl stearate	1.1	2.3	3.8
Methyl eicosanoate	1.8	3.8	6.0
Methyl docosanoate	2.9	6.2	9.8

Conditions: Columns were all 5 ft. stainless steel, 4 mm i.d., He flow rate was 69 ml/min, temperature 268°, head pressures above atmospheric for 5, 15 and 25% were 17.8, 9.8 and 9.4 p.s.i. respectively, exit pressure was atmospheric.

Table II lists the retention times for a variety of substances and the conditions under which they were run. Of interest is the fact that squalane has a considerably lower retention time than squalene, while the latter has a retention time approximately that of a straight chain C_{30} hydrocarbon. The fact that perhydrolycopene can be separated cleanly from perhydro-β-carotene should be of interest to workers in the field of carotenoids.

Such rapid retention times should make the study of many naturally occurring waxes easier. By collecting fractions and saponifying them individually it should be possible to get a better understanding of what alcohols are combined with what acids.

TABLE II

RETENTION TIMES FOR VARIOUS SUBSTANCES ON A SILICONE GUM COLUMN (5% LIQUID PHASE)

*Substance**	*Formula*	*Col. length*	*Temp. °C*	*Flow rate ml/min*	*Head pressure p.s.i.*	*Retention time in min***	*Remarks*
Hydrocarbons							
n-Tetracosane	$C_{24}H_{50}$	5′	278	75	20.5	1.4	All cleanly resolved
n-Hexacosane	$C_{26}H_{54}$	5′	278	75	20.5	2.4	
n-Octacosane	$C_{28}H_{58}$	5′	278	75	20.5	3.7	
n-Triacontane	$C_{30}H_{62}$	5′	278	75	20.5	5.9	
n-Hentriacontane	$C_{31}H_{64}$	5′	278	75	20.5	7.1	
n-Dotriacontane	$C_{32}H_{66}$	5′	278	75	20.5	9.2	
n-Tritriacontane	$C_{33}H_{68}$	5′	278	75	20.5	11.1	
n-Tetratriacontane	$C_{34}H_{70}$	5′	278	75	20.5	13.9	
Squalane	$C_{30}H_{62}$	5′	245	83	17	8.1	Cleanly separable
Squalene	$C_{30}H_{50}$	5′	245	83	17	12.1	
Squalene	$C_{30}H_{50}$	3′	270	87	18	2.2	
Triacontane	$C_{30}H_{62}$	3′	270	86	18	2.4	
Perhydrolycopene	$C_{40}H_{82}$	3′	270	85	18	9.7	
Perhydrolycopene	$C_{40}H_{82}$	20″	305	92	10	1.6	A well defined peak
Perhydrolycopene	$C_{40}H_{82}$	5′	282	61	12.5	11.3	Completely separated
Perhydro-β-carotene	$C_{40}H_{78}$	5′	282	61	12.5	16.6	

(Continued on p. 498)

TABLE II (*continued*)

*Substance**	*Formula*	*Col. length*	*Temp. °C*	*Flow rate ml/min*	*Head pressure p.s.i.*	*Rentention time in min***	*Remarks*
Methyl esters							
Myristate	$C_{15}H_{30}O_2$	5′	239	72	16.7	0.8	All cleanly resolved
Stearate	$C_{19}H_{38}O_2$	5′	239	72	16.7	2.4	
Eicosanoate	$C_{21}H_{42}O_2$	5′	239	72	16.7	4.3	
Docosanoate	$C_{23}H_{46}O_2$	5′	239	72	16.7	7.6	
Myristate	$C_{15}H_{30}O_2$	5′	287	71.5	18.9	0.2	All cleanly resolved
Stearate	$C_{19}H_{38}O_2$	5′	287	71.5	18.9	0.6	
Eicosanoate	$C_{21}H_{42}O_2$	5′	287	71.5	18.9	1.0	
Docosanoate	$C_{23}H_{46}O_2$	5′	287	71.5	18.9	1.5	
Tetracosanoate	$C_{25}H_{50}O_2$	5′	287	71.5	18.9	2.6	
Waxes							
Decyl palmitate	$C_{26}H_{52}O_2$	5′	277	75	20.5	3.8	All cleanly resolved
Dodecyl palmitate	$C_{28}H_{56}O_2$	5′	277	75	20.5	5.7	
Tetradecyl palmitate	$C_{30}H_{60}O_2$	5′	277	75	20.5	9.1	
Hexadecyl palmitate	$C_{32}H_{64}O_2$	5′	277	75	20.5	13.7	
Hexadecyl palmitate	$C_{32}H_{64}O_2$	3′	256	95	17.9	9.4	
Hexadecyl palmitate	$C_{32}H_{64}O_2$	20″	307	92	10	0.9	Sharp peak
Miscellaneous							
Ergosterol	$C_{28}H_{44}O$	3′	276	86	17.3	3.9	Some overlapping of peaks
Cholesterol	$C_{27}H_{46}O$	3′	276	86	17.3	3.5	
Ergosterol	$C_{28}H_{44}O$	5′	301	35	7.8	5.1	Some overlapping of peaks
β-Sitosterol	$C_{29}H_{50}O$	5′	301	35	7.8	6.5	
3β-Cholesteryl chloride	$C_{27}H_{45}Cl$	5′	250	86	20.2	11.6	
Cholestanone	$C_{27}H_{46}O$	5′	299	97	11.6	2.4	
α-Tocopherol	$C_{29}H_{50}O_2$	5′	278	75	20.5	7.4	Small overlap of peaks
γ-Tocopherol	$C_{28}H_{48}O_2$	5′	278	75	20.5	9.0	

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* All normal hydrocarbons were a gift of the Shell Oil Co., Amsterdam, The Netherlands, squalane and squalene were commercial products (Distillation Products, Inc.), perhydrolycopene and perhydro-β-carotene, a gift of Dr. O. ISLER of the Hoffmann LaRoche Co., Basle, Switzerland. The methyl esters were prepared by distillation of commercial products through a Podbielniak distillation column as were also the alcohols used in the preparation of the synthetic waxes. The sterols and sterol derivatives were a gift of Dr. H. WERBIN, and the tocopherols were purchased from Distillation Products, Inc.

Each compound was tested for chromatographic homogeneity by running it individually before testing it in mixtures.

** Bracketed compounds were run as mixtures.

The sterols generally showed broader peaks but separations are still possible in many instances especially where they differ by one carbon atom or have functional groups other than the OH group. Where the latter is the case the peaks are usually sharper.

Gas chromatographic analysis of sterols at 287° with columns of 20 % by weight silicone oil on Celite 545 has recently been reported[7].

The situation with regard to the tocopherols is similar to that of the sterols. α-Tocopherol can be separated nearly completely from γ-tocopherol and there is no reason to suppose that these could not be separated from δ-tocopherol, $C_{27}H_{46}O_2$.

It should be pointed out that the silicone rubbers and grease as used here will not separate unsaturated fatty acid methyl esters. However, they do separate fatty esters according to carbon number. The agreement between weight percentage and area percentage is within ± 1.3 % (see Table III) in contrast to the polyesters where correction factors have to be employed[8].

TABLE III

CORRESPONDENCE BETWEEN WEIGHT AND AREA PERCENTAGES OF METHYL ESTERS ANALYZED BY GAS CHROMATOGRAPHY ON A 5% SILICONE RUBBER COLUMN

	Area %	*Weight* %	*Difference*
Methyl myristate	16.6	16.1	0.5
Methyl stearate	31.4	29.3	2.1
Methyl eicosanoate	27.0	27.3	0.3
Methyl docosanoate	25.1	27.3	2.2
	Average deviation		1.28

Conditions: See Table I.

Acknowledgement

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Note added in proof

Since this article went to press, two communications have appeared in which are described separations of steroids[a] and alkaloids[b] by means of essentially the same system as employed here:

[a] W. J. A. VANDENHEUVEL, C. C. SWEELEY AND E. C. HORNING, *J. Am. Chem. Soc.*, 82 (1960) 3481.
[b] H. A. LLOYD, H. M. FALES, P. F. HIGHET, W. J. A. VANDENHEUVEL AND W. C. WILDMAN, *J. Am. Chem. Soc.*, 82 (1960) 3791.

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The detection of non-ketonic steroids on paper chromatograms*

The separation and isolation of non-ketonic steroid compounds by paper chromatography has been handicapped by the lack of adequate methods for their detection. The method to be described depends on the oxidation of hydroxyl groups to ketonic functions which can form color products with the Zimmerman reagent. This method has been applied to the localization and separation of C_{19}, C_{19} nor and C_{21} compounds. After chromatography the lower limits of sensitivity were found to be between 20–40 μg/cm² of paper. The method is as follows:

1. The dried paper chromatograms are passed quickly through a freshly prepared solution of 0.25 % CrO_3 in 90 % acetic acid. The strips are blotted lightly and placed in an oven at 90° for 15 min.

2. The strips are then developed with a modified Zimmerman reagent[1] as follows. The strips are first passed through a solution of 2.5 *N* KOH in absolute ethanol, blotted thoroughly and passed through a solution of 2 % *m*-dinitrobenzene in ethanol, again thoroughly blotted and placed in an oven at 60° for 1–2 min to develop the color. The colored zones should be marked as soon as possible since some of the color products tend to fade very rapidly.

The compounds listed in Table I were spotted on paper and treated as described above.

TABLE I

Compound	*Color after oxidation and Zimmerman*
Androstane-3α,17β-diol	Purple
Androstane-3β,17β-diol	Purple
Etiocholane-3α,17β-diol	Purple
Δ^{16}-Androsten-3β-ol	Blue fading to tan
Δ^{16}-Androsten-3α-ol	Blue fading to tan
19-Nor-5α-androstane-3β,17β-diol	Purple
19-Nor-5α-androstane-3α,17β-diol	Purple
19-Nor-5β-androstane-3α,17β-diol	Purple
19-Nor-5β-androstane-3β,17β-diol	Purple
Allopregnane-3α,20β-diol	Blue fading to tan
Pregnane-3α,20α-diol	Blue fading to tan
Pregnane-3α,17α,20α-triol	Purple
β-Allocortol	Purple
Pregnanolone	Blue fading to tan
Pregnane-3α,20α-diol, diacetate	Negative

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* Supported in part by U. S. P. H. S. grant No. A-2672 and U. S. P. H. S. Training Grant CRTY-5001.

The method has been successfully applied to the detection, separation and isolation of the following pairs of compounds after paper chromatography:

(a) Etiocholane-3α,17β-diol and androstane-3α,17β-diol
(b) Δ^{16}-Androsten-3α-ol and Δ^{16}-androsten-3β-ol
(c) 19-Nor-5α-androstane-3β,17β-diol and 19-nor-5α-androstane-3α,17β-diol
(d) 19-Nor-5β-androstane-3α,17β-diol and 19-nor-5β-androstane-3β,17β-diol

Similarly pregnane-3α,17α,20α-triol has been chromatographed on paper and could be detected at levels down to 30 μg/cm² of paper.

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[1] HARRIS ROSENKRANTZ, personal communication.

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Use of a diaphragm type burette tap and a constant level device for flow rate control in ion exchange chromatography

Since it is essential in ion exchange chromatography that a close approach to equilibrium conditions shall prevail in the column, it is imperative that the flow rate be kept as low and constant as possible[1,2]. A flow rate of ~ 1 ml·sq.cm^{-1}·min^{-1} is commonly employed in ion exchange chromatography[1].

The rate of flow is usually regulated by adjusting the height of the reservoir containing the eluant[1,3,4], or by means of a glass stopcock or pinchclamp[1,3]. Unless a reservoir of large volume is used, it is difficult to ensure a constant flow rate without continually adjusting the height of the reservoir. Glass stopcocks and pinchclamps are unsuitable for obtaining steady flow rates.

The following arrangement has been successfully employed for obtaining slow, steady flow rates during the HCl elution chromatographic enrichment of trace constituents in silicate rocks and allied materials[5–7]. A Townson and Mercer Series II diaphragm type polyethylene burette tap was attached to the lower end of the cation exchange column by means of a short length of Tygon tubing. A constant head of eluant was kept on the column by means of a "constant level" device constructed from a 1 l polyethylene bottle having a 10 cm long and 0.5 cm diameter polyethylene tube inserted into a hole drilled in the screw cap of the bottle. The tip of the polyethylene tube was bevelled at an angle of 45°. The column head was filled $^2/_3$ full with the desired eluant and the constant level device filled $^3/_4$ full of eluant was inverted and the bevelled tip of the polyethylene tube was inserted just below the surface of the liquid in the chromatography tube. When the liquid level dropped below the bevelled tip of the polyethylene tube an air bubble entered the bottle and allowed an equal volume of acid to flow into the column head.

A flow rate of 0.05 ml·sq.cm^{-1}·min^{-1}. could be readily obtained using the above arrangement with a uniformly packed bed of narrow sieve range of resin particles. Over a 12 hour period the variation in flow rate was ∼ 5 %.

Since avoidance of contamination is essential in trace analysis, polyethylene, on account of its general freedom from metallic contamination[8], was ideally suited for the construction of the apparatus described above.

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Gaschromatographische Reinheitsbestimmung der Malonester

Für Malonestersynthesen wird zum grossen Teil auch Monomethylmalonester verwendet, der durch die Synthese mit Dimethylmalonester und Malonester verunreinigt ist. Es ist sehr schwierig, in grösserem Masstab Methylmalonester herzustellen, der frei von Malonester und Dimethylmalonester ist. Durchgeführte gaschromatographische Untersuchungen, deren Ergebnis hier mitgeteilt werden soll, haben gezeigt, dass die Reinheit von Methylmalonester gaschromatographisch geprüft werden kann.

Arbeitsbedingungen. Gaschromatograph: Fraktometer Mod. 116 der Fa. Perkin-Elmer, Überlingen/Bodensee, Thermistorzelle, Temperatur 180°, Säulendruck 1.55 atü, Detektorspannung 8.0 V; Säule: Silikonfett (Dow Corning Corporation, Silicone DC Hochvakuumfett, Wacker Chemie GmbH) auf Celite 535 (Johns-Manville) 0.2–0.5 mm, Mischungsverhältnis 3:10, Säulenlänge 10 m, V2A-Rohr 4 mm lichte Weite; Trägergas: Wasserstoff.

Fig. 1 a zeigt die nach diesen Arbeitsbedingungen erzielte Trennung der drei Ester. Am bemerkenswertesten ist bei dieser Trennung die Verwendung von Celite 535, da mit keinem anderen Träger eine Trennung erzielt werden konnte. In Fig. 1 b ist dieselbe Trennung unter den gleichen Bedingungen mit Silikonfett auf Chromosorb wiedergegeben. Dieser Befund der Überlegenheit von Celite 535 (oder Celite 545) gegenüber Chromosorb und anderen Trägern bei der gaschromatographischen Tren-

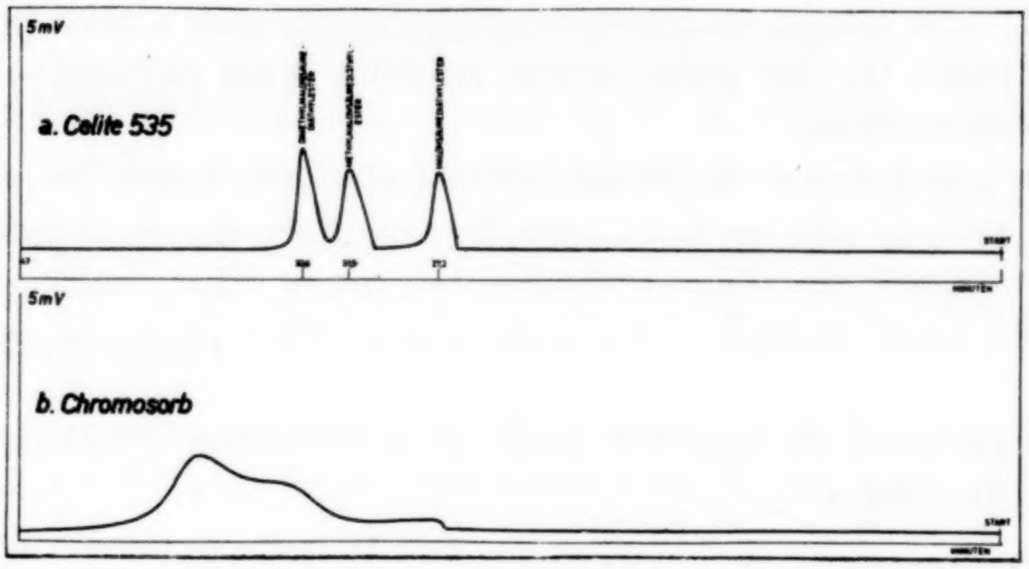

Fig. 1. GC-Trennung der Malonester an Silikonfett auf Celite 535 und Chromosorb.

nung polarer Substanzen wurde auch bei anderen Trennungen gemacht, insbesondere bei der Trennung von Wasser und Fettsäuren, Wasser und Alkoholen und Wasser und Aldehyden.

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Eingegangen den 5. August 1960

BOOK REVIEWS

Handbook of Filtration, by the Technical Staff of the Eaton-Dikeman Company, The Eaton-Dikeman Company, Mount Holly Springs, Pa., 1960, 124 pages, 45 ill., price $ 2.50.

According to the dust-jacket summary, this book has been directed towards three classes of people of differing interests: the laboratory worker, the chemical engineer and "those persons who have a general interest in filtration".

To write for three groups of differing interests and at the same time to satisfy the respective interests is not an easy task and this handbook has not succeeded in attaining these ends.

Probably the chemical engineer will find much to interest him but the laboratory worker will be disappointed. The chapter on retention is the most interesting from the laboratory technician's view point. The reviewer would have liked to see the standard blocking concept developed more fully and some indication whether this effect varies with the nature of the substance being filtered and if so, what is the size range of particles retained by this method? The section on laboratory applications reads more like a popular science account than a Handbook of Filtration. The sprinkling of paper maker's jargon is not always clearly explained in the glossary, *e.g.* the definition

of "formation". The section on chromatography opens with a most inaccurate definition of the term; the one given covers anything from physical separation of a mixture to group analysis.

It is to be hoped that in the supplementary chapters, which are promised in the foreword, the authors will see fit to include some quantitative information on the swelling of filter paper, the amount of solvent (water or other) which a given type of paper will hold when saturated, the resistance of filter paper to acid or alkaline solutions.

In its present form the book is of more use to the college chemistry student than to the laboratory worker.

T. J. BECKMANN (Brisbane)

Inorganic Chemistry: A Guide to Advanced Study, by R. B. HESLOP AND P. L. ROBINSON, Elsevier Publ. Co., Amsterdam, 1960, 556 pages, price 45 s, Dfl. 24.00.

The authors have set themselves the task of presenting the whole field of inorganic chemistry from the modern point of view. The historical development of the atomic model of Bohr and of the periodic table are intentionally omitted. The topics dealt with fall essentially into two sections, those which deal with general things such as radiochemistry, the ligand field theory, chemical kinetics, etc., and those which discuss the chemistry of elements.

For the first section it must be said that they excel in clarity and briefness. Most topics are discussed in short paragraphs or chapters but are extremely well written so that it is possible, for example, to get the essentials of radiochemistry by reading only eleven pages. The essentials of chemical thermodynamics and chemical kinetics, usually considered part of physical chemistry, receive an equally good and brief treatment.

In the chemistry of the elements, the main emphasis is placed on structural chemistry, electronic configuration and industrial preparations, but is rather unbalanced as regards the solution chemistry of ions. Occasionally problems of the solution chemistry are mentioned in greater detail than usually found in textbooks, but very often the amount of information is inadequate, for example the list of charge numbers of the platinum metals or the chemistry of the first four actinides. It is also surprising that the authors have mentioned the colours of substances only very occasionally. However, these shortcomings can be overcome if the book, which is of course meant as a textbook, is combined with a lecture course which features numerous demonstrations.

The book should be read by everybody who teaches or studies inorganic or general chemistry.

The Bibliography of Chromatography

Gas Chromatography Abstracts 1959, edited by C. E. H. KNAPMAN, Butterworths Sci. Publ., London, 1960, ix + 164 pages, price 42 s.

Handbuch der Papierchromatographie, Band II, *Bibliographie und Anwendungen*, by I. M. HAIS AND K. MACEK, VEB Gustav Fischer Verlag, Jena, 1960, x + 726 pages, price DM 44.—.

Bibliografie Papírové Chromatografie, a Přehled Použití, by I. M. HAIS AND K. MACEK, Nakladatelství Československé Akademie Věd, Praha, 1960, 766 pages, price Kcs 91.—.

The literature on chromatography has become so extensive that the publication of collected bibliographies was found necessary.

The *Gas Chromatography Abstracts 1959* is the second volume compiled by the abstracting committee of the Gas Chromatography Discussion Group. They list 717 references which have appeared in 1959. Although there is no classification in the sequence of the references, a detailed subject index (33 pages) enables the reader to find his bearings rapidly.

The bibliography of paper chromatography by HAIS AND MACEK appeared simultaneously in Czech and German. It covers the literature up to 1956 listing 10,290 references, which are divided into chapters according to the substances chromatographed. The general section deals also with the history, reviews and books, theory and technique. Each reference gives the complete title of the paper in its original language. Thus except for the titles of the chapters both the Czech and the German edition can be used by readers without a knowledge of these two languages.

The authors of these bibliographies have performed a very important task. Since all handbooks and reviews on chromatography nowadays deal either with a selection of the literature or with a specialized field such bibliographies will be invaluable for all laboratories.

J. Chromatog., 4 (1960) 505

The Bibliography of Electrophoresis

Journal of Electroanalytical Chemistry, International Journal Dealing with all Aspects of Electroanalytical Chemistry, Including Fundamental Electrochemistry. Editors: G. CHARLOT, J. O'M. BOCKRIS, G. MILAZZO AND C. N. REILLEY. Vol. 1 (1959–60), Elsevier Publ. Co., Amsterdam.

The need to collect the literature of paper chromatography and gas chromatography was realized recently and reference lists on these topics have appeared in book form. The field of paper electrophoresis is perhaps still more difficult to survey as its literature is found in chemical as well as medical journals. It seems hence very opportune that the *Journal of Electroanalytical Chemistry* has started an *Abstracts Section* dealing with all electroanalytical methods including a section on electrophoresis.

This abstracts section "... begins with abstracts of papers published since January 1st 1959 and an attempt will be made to cover all papers of interest to readers of the Journal published in 1959 and 1960 as rapidly as possible. The Editors hope to achieve this objective about the middle of 1961, and from then on current abstracting of papers within about two or three months after publication should be realized."

It is obvious that this project neither competes with nor duplicates the work of the *Chemical Abstracts*, which on the one hand claim to be complete but on the other appear a long time after the original publication.

The first lot of abstracts has appeared in the May 1960 issue of the journal and lists 120 abstracts on electroanalytical topics including seven on electrophoresis.

J. Chromatog., 4 (1960) 505–506

NEW BOOKS

Chromatografia, revision de sus principios y aplicaciones, by E. LEDERER AND M. LEDERER, El Ateneo, Buenos Aires, 1960, xix + 752 pages.

A Spanish translation of the second English edition, by FREDERICO VILALLONGA CORDOMI.

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Instability Constants of Complex Compounds, by K. B. YATSIMIRSKII AND V. P. VASIL'EV, Pergamon Press, London, 1960, approx. 230 pages, price 60 s.

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CHROMATOGRAPHIC DATA

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TABLE 1

R_F VALUES OF SOME NITRO-DERIVATIVES OF SULPHAMIDOBENZOIC ACIDS

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 3881)

Solvent: *n*-Butanol–pyridine–water (5:3:3).
Paper: Whatman No. 4 (descending).
Length of run: 23 cm.
Time: 4h.
Temperature: 24°.
Detection: Ehrlich reagent after Zn(II)-chloride reduction.

Benzoic acid derivative	R_F
3-Nitro-4-sulphamido-	0.64
2-Nitro-4-sulphamido-	0.95
4-Nitro-2-sulphamido-	0.75
6-Nitro-2-sulphamido-	0.65
5-Nitro-2-sulphamido-	0.96
3,5-Dinitro-4-sulphamido-	0.28
2,3-Dinitro-4-sulphamido-	0.17
2,5-Dinitro-4-sulphamido-	0.75
4,6-Dinitro-2-sulphamido-	0.75
3,6-Dinitro-2-sulphamido-	0.38
2,5,6-Trinitro-4-sulphamido-	0.71
2,3,5-Trinitro-4-sulphamido-	0.33
3,4,6-Trinitro-2-sulphamido-	0.38

TABLE 2

ELECTROPHORETIC MOBILITIES OF SOME NITRO-DERIVATIVES OF SULPHAMIDOBENZOIC ACIDS AND RELATED SUBSTANCES

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 3881)

Electrolyte: 3 N NH_4OH (pH $>$ 10).
Paper: Whatman No. 1.
Potential: 6–7 V/cm.
Current: 2–6 mA.
Time of run: 2 h.
Migration units: $cm^2 \cdot V^{-1} \cdot sec^{-1} \times 10^5$ (mobility). Anionic movement: +; cationic movement: —.

Compound	*Mobility*
3-Nitrophthalic acid*	+ 20.5
o-Toluenesulphamide	— 2.8
p-Toluenesulphamide	— 2.8
4-Sulphamido-3-nitrobenzoic acid	+ 8.4
2-Sulphamido-4-nitrobenzoic acid	+ 8.4

* Marker.

TABLE 3

ELECTROPHORETIC MOBILITIES OF AROMATIC COMPOUNDS

(J. FRANC AND M. WURST, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 857)*

Electrolytes: $E_1 = 3\ N\ NH_4OH$ (pH > 10).
$E_2 = 1\ N\ CH_3COOH$ (pH 2.3).
Paper: Whatman No. 1 (13 × 45 cm).
Potential: 6–7 V/cm.
Current: 2–6 mA.
Time of run: 2 h.
Migration units: $cm^2 \cdot V^{-1} \cdot sec^{-1} \times 10^5$ (mobility).

Compound	*Mobility***	
	E_1	E_2
Acids		
3-Nitrophthalic acid	20.5	6.4
4-Nitrophthalic acid	20.5	5.0
2-Nitroterephthalic acid	20.5	6.3
5-Nitroisophthalic acid	21.1	3.3
3-Nitrobenzoic acid	11.6	1.3
4-Nitrobenzoic acid	11.6	1.4
3,5-Dinitrobenzoic acid	11.1	1.9
2,4,6-Trinitrobenzoic acid	10.0	8.8
2,4,5-Trinitroterephthalic acid	20.3	—
3,5-Dinitro-*o*-toluic acid	11.4	—
3,5-Dinitro-*p*-toluic acid	11.4	—
2,3,5-Trinitro-*p*-toluic acid	11.1	—
3-Nitro-*p*-toluic acid	11.4	—
2,4-Dinitro-*m*-toluic acid	11.6	—
2-Aminobenzoic acid	12.3	1.8
3-Aminobenzoic acid	11.5	6.2
4-Aminobenzoic acid	11.6	3.0
2-Hydroxybenzoic acid	15.5	2.7
4-Hydroxybenzoic acid	20.3	0.9
Gallic acid	19.5	0.6
2-Hydroxy-3-methoxybenzoic acid	13.5	2.8
Aldehydes		
3-Methoxy-4-hydroxybenzaldehyde	10.7	0.7
3,4-Dimethoxybenzaldehyde	0.5	1.4
2-Hydroxy-3-ethoxybenzaldehyde	11.8	1.6
3-Hydroxy-4-methoxybenzaldehyde	11.7	1.3
2-Nitrobenzaldehyde	0.0	0.0
3-Nitrobenzaldehyde	0.0	0.0
4-Nitrobenzaldehyde	0.0	0.0
4-Chlorobenzaldehyde	0.0	0.0
4-Methoxyacetophenone	0.0	0.4
Amines		
2-Nitraniline	0.0	−0.8
3-Nitraniline	0.0	−3.9
4-Nitraniline	0.0	−0.6
2,4-Dinitraniline	0.0	0.0
1-Naphthylamine	−0.7	−10.4
p-Phenylenediamine	−3.7	−20.6

(continued on p. D3)

TABLE 3 (*continued*)

Compound	*Mobility*[**]	
	E_1	E_2
Phenols		
2-Nitrophenol	12.5	0.0
3-Nitrophenol	11.4	0.0
4-Nitrophenol	11.3	0.0
2,4-Dinitrophenol	10.1	0.0
2,6-Dinitrophenol	12.0	0.0
4,6-Dinitro-*o*-cresol	8.7	0.7
5-Nitro-1,3,4-xylenol	11.4	—
2,4,6-Trinitroresorcinol	17.2	7.4
4,6-Dinitro-2-aminophenol	9.1	—
1-Naphthol	9.6	0.0
Resorcinol	18.4	0.0
Nitroderivatives		
1,3-Dinitrobenzene	0.0	0.0
3,4,6-Trinitro-*o*-xylene	0.0	0.0
2,4,6-Trinitro-*m*-xylene	0.0	0.0
2,3,5-Trinitro-*p*-xylene	0.0	0.0
1,4-Dichloro-2-nitrobenzene	0.0	0.0
2,6-Dichloro-4-nitrobenzene	0.0	0.0
Naphthylamine- and naphthol-sulphonic acids		
1-Naphthylamine-4-sulphonic acid	9.3	4.5
1-Naphthylamine-5-sulphonic acid	9.5	0.0
1-Naphthylamine-6-sulphonic acid	8.9	0.0
1-Naphthylamine-7-sulphonic acid	8.9	0.0
1-Naphthylamine-8-sulphonic acid	9.5	0.0
2-Naphthylamine-1-sulphonic acid	9.6	6.2
2-Naphthylamine-6-sulphonic acid	9.3	0.0
2-Naphthylamine-5-sulphonic acid	9.3	0.0
2-Naphthylamine-7-sulphonic acid	9.3	0.0
1-Naphthylamine-3,6-disulphonic acid	20.4	13.4
1-Naphthylamine-3,6,8-trisulphonic acid	26.6	14.9
1-Naphthol-4-sulphonic acid	20.0	7.1
1-Naphthol-5-sulphonic acid	20.1	7.2
2-Naphthol-6-sulphonic acid	19.4	7.0
2-Naphthol-8-sulphonic acid	20.3	7.2
2-Naphthol-3,6-disulphonic acid	24.0	13.0
2-Naphthol-6,8-disulphonic acid	25.3	13.0
1-Naphthol-3,6,8-trisulphonic acid	—	17.2
H-acid	25.8	11.7
Chromotropic acid	27.3	10.5

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[*] First published in *Chem. listy*, 52 (1958) 1474.

[**] Anionic movement: +; cationic movement: —. No correction for electrolyte movement through the paper as a result of electroendosmosis, evaporation etc.

TABLE 4

R_F VALUES OF SOME CHLORONITRONAPHTHALENES

(J. FRANC, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 3624)

Solvents: Stationary phases:
S_1 = Liquid paraffin (boiling range: 190–275°) (impregnated as 20% solution in petroleum ether).
S_2 = Diethylformamide.
S_3 = Dimethylformamide.
S_4 = Acetamide.
(S_2–S_4 impregnated as 30% solutions in ethanol.)
Mobile phases:
M_1 = 80% ethanol.
M_2 = Cyclohexane.
Time of run: S_1, 16 h; S_2–S_4, 3 h.
Length of run: 30 cm.
Paper: Whatman No. 1 (descending).
Detection: U.V.; *p*-dimethylaminobenzaldehyde after $ZnCl_2$ reduction.

Naphthalene or benzene derivative	R_F			
	M_1	M_2		
	S_1	S_2	S_3	S_4
1-Nitro-8-chloronaphthalene	0.76	0.18	0.49	0.83
1-Nitro-5-chloronaphthalene	0,46	0.44	0.79	0.92
1-Nitro-5,8-dichloronaphthalene	0.57	0.33	0.68	0.88
1-Nitro-4,5,8-trichloronaphthalene	0.39	0.49	0.80	0.91
2-Nitro-8-chloronaphthalene	0.49	0.42	0.76	0.89
2-Nitro-4-chloronaphthalene	0.91	0.08	0.22	—
2-Nitro-4,8-dichloronaphthalene	0.27	0.61	0.85	0.93
3,4-Dichloronitrobenzene	0.52	0.51	0.80	0.93
2,5-Dichloronitrobenzene	0.65	0.25	0.43	0.54
1-Nitronaphthalene	0.65	0.32	0.67	0.89
2-Nitronaphthalene	0.67	0.29	0.65	0.90

TABLE 5

R_F VALUES OF SOME CHLOROPHENOLS

(D. V. PARKE AND R. T. WILLIAMS, *Biochem. J.*, 74 (1960) 5)

Solvents: S_1 = Benzene–acetic acid–water (1:1:2, by vol.).
S_2 = Ethanol–butan-1-ol–aq. 6 *N* NH_3 soln.–6 *N* $(NH_4)_2CO_3$ (22:80:19:19, by vol.).
S_3 = *n*-Hexane–diisopropyl ether (9:1, v/v).
Paper: Whatman No. 1 for S_1 and S_2 (descending).
Whatman No. 4 impregnated with aq. 0.2 *N* Na_2CO_3 for S_3 (descending).
Time of run: S_1 for 6 h; S_2 for 12 h; S_3 for 0.5 h.
Detection: Gibbs reagent (2% ethanolic solution of 2,6-dichloroquinone chloroimide followed by 2 *N* Na_2CO_3; diazotized sulphanilic acid, followed by 2 *N* Na_2CO_3; aq. 1% $FeCl_3$, followed by saturated $NaHCO_3$; 5 *N* HNO_3 followed by aq. 2% urea after heating the dried paper (2 min, 120°) followed by solution of 0.5 g tetramethyl-*p,p'*-diaminodiphenylmethane (Tetrabase) and 2 g citric acid in 100 ml water.

Phenol	R_F		
	S_1	S_2	S_3
4-Chlorophenol	0.78	0.91	0.84
2,4,6-Trichlorophenol	0.92	—	0.10
Pentachlorophenol	0.92	0.85	0.04
4-Chlorocatechol	0.33	—	0.06

TABLE 6

R_F VALUES OF SOME HYDROXYNAPHTHALENE DERIVATIVES AND RELATED COMPOUNDS

(P. SIMS, *Biochem. J.*, 73 (1959) 389)

Solvents: S_1 = *n*-Butanol saturated with aqueous 2 *N* NH_3 solution (descending).
S_2 = *n*-Butanol–propan-1-ol–aqueous 2 *N* NH_3 solution (2:1:1, by vol.) (descending).
S_3 = Aqueous 0.1 *N* NH_3 solution (ascending).
Time of run: S_1 and S_2, 15 h; S_3, 5 h.
Paper: Whatman No. 1.
Detection: U.V. after NH_3 exposure; diazotized *p*-nitraniline (0.02% in 0.1 *N* HCl), then 10% aqueous Na_2CO_3 solution (or after aqueous 2 *N* HCl (70°)).

Compound	R_F		
	S_1	S_2	S_3
1-Naphthol	0.96	0.97	0.62
2-Naphthol	0.96	0.97	0.55
trans-1,2-Dihydro-1,2-dihydroxynaphthalene	0.86	0.89	—
1-Naphthyl sulphate	0.51	0.62	—
2-Hydroxy-1-naphthyl sulphate	0.49	0.58	—
1-Hydroxy-2-naphthyl sulphate	0.46	0.55	—
2-Naphthyl sulphate	0.46	0.53	—
1-Naphthyl-glucosiduronic acid	0.11	0.28	—
1,2-Dihydro-2-hydroxy-1-naphthyl-glucosiduronic acid*	0.08	0.18	—
1,2-Dihydro-1-hydroxy-2-naphthyl-glucosiduronic acid	0.08	0.18	—

* The optically active forms could not be distinguished on paper chromatograms.

TABLE 7

R_F VALUES OF COMPOUNDS RELATED TO S-(1,2-DIHYDRO-2-HYDROXY-1-NAPHTHYL)-GLUTATHIONE

(J. BOOTH, E. BOYLAND AND P. SIMS, *Biochem. J.*, 74 (1960) 117)

Solvents: S_1 = Butanol–propan-1-ol–aq. 2 *N* NH_3 soln. (2:1:1, v/v).
S_2 = Butanol–acetic acid–water (2:1:1, v/v).
S_3 = Butanol–acetic acid–water (5:1:4, v/v organic phase).
Paper: Whatman No. 1 (descending).
Time of run: 18 h.
Detection: (1) Diazotized *p*-nitraniline (0.02% in 0.1 *N* HCl) followed by aqueous 10% Na_2CO_3 w/v; (2) 0.1 *M* $K_2Cr_2O_7$–acetic acid (1:1) followed by 0.1 *M* $AgNO_3$; (3) 0.4% ninhydrin in butanol saturated with water. Also before (1) some sprayed with 2 *N* HCl then heated at 70° (10 min).

Compound	R_F		
	S_1	S_2	S_3
S-(1,2-Dihydro-2-hydroxy-1-naphthyl)-glutathione	0.04	0.47	0.36
S-(1,2-Dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0.31	0.63	0.53
N-Acetyl-S-(1,2-dihydro-2-hydroxy-1-naphthyl)-L-cysteine	0.37	0.84	0.82
S-(1-Naphthyl)-glutathione	0.07	0.69	0.53
S-(1-Naphthyl)-L-cysteine	0.46	0.75	0.69
1-Naphthylmercapturic acid	0.51	0.86	0.87

TABLE 8

R_F VALUES OF SOME PHENOLIC COMPOUNDS

(C. MADHOSINGH, *Ph. D. Thesis*, University of Western Ontario, London, Canada, 1959)

Paper: Whatman No. 1.
Temperature: about 24°.
Abbreviations used in the table:

Solvents:

A = Pentanol–ethanol–ammonia solution (sp. gr. 0.88)–benzene (1:2:1:1). Used in an ascending system under reduced pressure.
B = Benzene–glacial acetic acid–water in a 1:1:2 volume to volume mixture. The organic phase was used in descending systems.
C = 1-Butanol saturated with water and used in an ascending system.

Detecting agents:

1 = Ultraviolet.
2 = Solvent A without further treatment.
3 = Solvent B without further treatment.
4 = Solvent C without further treatment.
5 = Diazotized sulfanilic acid.
6 = Silver nitrate 0.1 *N* in 8% NH_3 solution.
7 = 1 *N* sodium hydroxide.

Colour code:

a = Yellow.
b = Orange-yellow.
c = Dark violet.
d = Violet.
e = Light blue.
f = Flawn-brown.
g = Scorched paper brown-black.
h = Flame red-brown.
i = Silver-gray.
j = Green-gray.
k = Brown-gray.
l = Pink.
m = Orange.
(—) Indicates no apparent colour reaction with this detecting agent.

Compound	*RF × 100 in solvent systems*			*Colours with detecting agents*						
	C	*B*	*A*	*1*	*2*	*3*	*4*	*5*	*6*	*7*
2,4-Dinitrophenol	56	85	65	c	a	a	a		—	b
2-Amino-4-nitrophenol	86	100	52	c	b		a			b
4-Amino-2-nitrophenol	80	100	40	d	f	f	t			l
2,4-Diaminophenol	12		30	h	h	—	f		g	f
p-Nitrophenol	84	60	36	e	a	a	—		b	a
m-Nitrophenol	84	60	80	—	—			a	b	m
o-Nitrophenol	84	87	56	c	—		—	b	—	b
p-Aminophenol	72	5	71	e	f		f	j	g	f
m-Aminophenol	70	22	72	e	—		—	b	g	f
Picric acid	52		70	c	a		a		—	b
Picramic acid		6	30	c		—		—	g	—
Picramide	80	83	87		a	a		a		
Aniline	30		66	e		—		—	f	
Resorcinol	73	15	73	e				a	g	k
Dye of 2-amino-4-nitrophenol*	91	100	88	—	l		l	—		l
Dye of 4-amino-2-nitrophenol*	90	100	74	d	f		f			l
2,4-Diaminophenol-HCl**										
Spot 1	12		29		b		l			f
Spot 2	3		20	h	f		l			—
Spot 3	0	0	10		k		g			g

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* Diazotized and coupled with β-naphthol.
** Solution chromatographed after standing for 72 hours.

TABLE 9

R_F VALUES OF SOME RELATED ALKYL PHENOLS

(J. MACÁK AND J. KUBEŠ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 301)

Solvent: Cyclohexane.
Paper: WF-1 Paper (G.D.R.).
Impregnation: 20% alcoholic formamide.
Time of run: 4-6 h.
Temperature of run: 15–17°.
Detection: Pauly reagent.

Compound	R_F
Phenol	0.03
o-Cresol	0.13
m-Cresol	0.08
p-Cresol	0.09
o-Ethylphenol	0.30
m-Ethylphenol	0.18
p-Ethylphenol	0.19
3-Propylphenol	0.45
2-Methyl-4-ethylphenol	0.57
4-Methyl-2-ethylphenol	0.61
2-Butylphenol	0.70
3-Butylphenol	0.47
4-Butylphenol	0.49
2,5-Diethylphenol	0.71
3,4-Diethylphenol	0.48
2-Ethyl-5-propylphenol	0.83
2-Ethyl-5-butylphenol	0.89
4-Methyl-2-benzylphenol	0.67

TABLE 10

R_F VALUES OF SOME ARYL SULPHATES

(A. J. GRIMES, *Biochem. J.*, 73 (1959) 723)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, v/v).
S_2 = Butan-2-ol–aq. 3 *N* NH_3 soln.–3 *N* $(NH_4)_2CO_3$ (4:3:3).
S_3 = Butan-1-ol–pyridine–aq. 3 *N* NH_3 soln. (4:3:3).
Paper: Whatman No. 1 (ascending; no equilibration).
Time of run: 16 h.
Detection: Ninhydrin; HCl vapour (10 min) followed by Pauly reagent spray, then NH_3 vapour exposure; radioactivity.

Compound	R_F		
	S_1	S_2	S_3
Phenyl sulphate	0.49	0.35	0.76
p-Cresyl sulphate	0.61	0.34	0.77
p-Ethylphenyl sulphate	0.70	0.62	0.78
p-Acetylphenyl sulphate	0.54	0.25	0.77
Tyramine O-sulphate	0.34	0.10	0.66

TABLE 11

R_F VALUES (RELATIVE) OF POTASSIUM *p*-NITROPHENYL [^{35}S]SULPHATE, *p*-AMINOPHENYL [^{35}S]SULPHATE AND SODIUM [^{35}S]SULPHATE

(K. S. DODGSON AND N. TUDBALL, *Biochem. J.*, 74 (1960) 154)

Solvent: Butan-1-ol–acetic acid–water (50:12:25).
Paper: Whatman No. 1 (descending).
Detection: Radioactivity measured by "Panax" monitor after location by "C. 100 Actigraph"; Ilford Industrial B X-ray film (21 day exposure).
R_{NPS} = R_F of compound/R_F of *p*-nitrophenyl sulphate.

Compound	*RNPS*
Potassium *p*-nitrophenyl [^{35}S]sulphate	1.00
p-Aminophenyl [^{35}S]sulphate	0.55
Sodium [^{35}S]sulphate	0.12

TABLE 12

R_F VALUES OF SOME β-CHLOROVINYL ALDEHYDES AND RELATED COMPOUNDS

(Z. ARNOLD AND J. ŽEMLIČKA, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2385)

Solvents: S_1 = Dibutyl formal–water.
S_2 = Cyclohexane–methanol.
S_3 = Diisopropyl ether–water.

Compound	R_F		
	S_1	S_2	S_3
β-Dimethylaminocinnamaldehyde	0.41		
β-Dimethylaminovinyl *tert.*-butyl ketone		0.55	
2-Dimethylaminocyclopent-1-enealdehyde	0.06		
2-Dimethylaminomethylenecyclopentanone	0.26		
β-Dimethylaminovinyl ethyl ketone			0.33
α-Methyl-β-dimethylvinyl methyl ketone			0.04

TABLE 13

R_F VALUES OF MEVALONIC ACID (3,5-DIHYDROXY-3-METHYLPENTANOIC ACID) AND SOME RELATED COMPOUNDS

(A. DE WAARD AND G. POPJÁK, *Biochem. J.*, 73 (1959) 410)

Solvent: Isobutyric acid–aqueous NH_3 solution–water (66:3:30, v/v).
Paper: Whatman No. 1.
Detection: U.V. photography, radioactivity scan.

Compound	R_F
Mevalonic acid	0.66
Mevalonic acid lactone	0.75
5-Phosphomevalonic acid*	0.37
Diphosphomevalonic acid*	0.20
Adenosine diphosphate	0.37
Adenosine triphosphate	0.20

* Tentative identification.

TABLE 14

R_F VALUES OF SOME PHOSPHOLIPIDS

(C. E. ROWE, *Biochem. J.*, 73 (1959) 438)

Solvent: 20% methanol in chloroform (v/v).
Paper: Silica impregnated.
Detection: Ninhydrin, iodine vapour, phosphomolybdic acid followed by $SnCl_2$ reduction; radioautography.

Compound	R_F
Cephalin	0.92
Lecithin	0.75
Sphingomyelin	0.55

TABLE 15

R_F VALUES (RELATIVE) OF SOME PHOSPHATE ESTERS

(S. A. BROOKS, J. C. LAWRENCE AND C. R. RICKETTS, *Biochem. J.*, 73 (1959) 566)

Solvents: S_1 = Propan-1-ol–aq. NH_3 soln. (sp. gr. 0.880)–water (60:30:10, by vol.).
S_2 = Picric acid–2-methylpropan-2-ol–water (4:80:20, w/v/v).
Paper: Whatman No. 1 (18¼ in. × 22½ in.) (descending; S_1 direction: run off; S_2 direction: front run to 2 in. from bottom). Paper soaked in 0.2% EDTA (pH 7.8), then water washed (two-dimensional runs).
Time of run: S_1 for 40 h; S_2 for 24 h.
Detection: Autoradiography (Kodirex X-ray film 14 in. × 17 in., 9 days); spray of mixture: 5 ml of 60% (w/w) perchloric acid, 10 ml of *N* HCl, 25 ml of 4% (w/v) ammonium molybdate, water to 100 ml; then heated at 85° for 7 min; then sprayed with 5% (w/v) ascorbic acid; aniline hydrogen phthalate. The suitable method chosen in each case.

$$R_I \text{ value} = \frac{\text{distance moved by phosphate ester}}{\text{distance moved by orthophosphate}}$$

Where more than one R_I value is given, the first in S_1 is paired with the first in S_2, etc.: the pair is from the same two-dimensional chromatogram (information from 41 chromatograms).

Compound	R_I	
	S_1	S_2
Orthophosphate	1.00	1.00
Fructose-6-phosphate	1.19, 1.26	0.64, 0.68
3-Phospho-D-glyceric acid	1.05, 1.16	0.87, 0.88
Uridine diphosphate	0.77, 0.78	0.35, 0.40
Uridine diphosphoglucose	1.29, 1.27	0.16, 0.19
Adenosine monophosphate	0.95, 1.01, 0.89	0.33, 0.41, 0.33
Mannose-6-phosphate	1.08	0.54
Glucose-6-phosphate	1.05, 1.09	0.46, 0.41
Adenosine diphosphate	0.95, 0.94, 0.89	0.22, 0.25, 0.21
Guanosine diphosphate	0.38	0.24
Phosphoenolpyruvic acid	1.13, 1.16	1.16, 1.09
Adenosine triphosphate	0.71, 0.66, 0.67	0.15, 0.12, 0.06
Fructose-1,6-diphosphate	0.40, 0.46	0.68, 0.78
Glucose-1-phosphate	1.28, 1.35, 1.33	0.51, 0.51, 0.41
Pyrophosphate	0.49, 0.52, 0.56	0.42, 0.45, 0.47
2-Deoxyglucose-6-phosphate	1.25, 1.34	0.79, 0.77
Glucosone-6-phosphate	1.25, 1.36	0.57, 0.59
1,5-Anhydro-D-glucitol-6-phosphate	1.31, 1.34	0.77, 0.70
Glucosamine-6-phosphate	1.32, 1.38	0.54, 0.57

TABLE 16

R_F VALUES OF SOME AZAURACIL DERIVATIVES AND RELATED COMPOUNDS

(J. SMRT, J. BERÁNEK AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 130)

Solvents: S_1 = *n*-Butanol saturated with water.
S_2 = *n*-Butanol–acetic acid–water (5:2:3).
S_3 = Isopropanol–1 % aqueous ammonium sulphate (2:1).
Paper: Whatman No. 1 (descending).
Detection: U.V. light ("Chromatolite"); ammonium molybdate–perchloric acid.

Compound	R_F		
	S_1	S_2	S_3
2′,3′-O-Isopropylidene-6-azauracil riboside	0.78		
2′,3′-O-Isopropylidene-6-azauracil riboside-5′-dibenzyl phosphate	0.88		
β-D-Ribofuranosyl-6-azauracil-5′-phosphate (Ba salt)	0.00	0.04	0.30
p-Nitrophenol	0.91		
β-D-Ribofuranosyl-6-azauracil-5′-dibenzyl pyrophosphate	0.72		

TABLE 17

R_F VALUES OF AZAURIDINE-5′-PHOSPHATE AND AZAURIDINE-5′-PYROPHOSPHATE

(J. SMRT, J. BERÁNEK AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 130)

Solvents: S_1 = *n*-Butanol–acetic acid–water (5:2:3).
S_2 = Isopropanol–1 % aqueous ammonium sulphate (2:1).
Paper: Whatman No. 1 (descending).
Detection: U.V. light ("Chromatolite"); ammonium molybdate–perchloric acid.

Compound	R_F	
	S_1	S_2
Azauridine-5′-phosphate	0.04	0.30
Azauridine-5′-pyrophosphate	0.00	0.16

TABLE 18

ELECTROPHORETIC MOBILITY OF AZAURIDINE-5′-PHOSPHATE AND AZAURIDINE-5′-PYROPHOSPHATE

(J. SMRT, J. BERÁNEK AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 130)

Electrolyte: Citrate buffer (0.05 *M*, pH 3.7).
Paper: Whatman No. 3.
Potential: 100 V/cm.
Units: cm/2 h.
Apparatus: Markham (1955).
Detection: U.V. light ("Chromatolite"); ammonium molybdate–perchloric acid.

Compound	*Mobility*
Azauridine-5′-phosphate	12.9
Azauridine-5′-pyrophosphate	20

TABLE 19

ELECTROPHORETIC MOBILITIES OF METHYLATED GUANOSINES

(J. D. SMITH AND D. B. DUNN, *Biochem. J.*, 72 (1959) 294)

Electrolytes: E_1 = 0.05 M sodium tetraborate (pH 9.2).
E_2 = 0.05 M glycine buffer, made by adding KOH to an aqueous glycine solution (pH 10).
E_3 = 0.05 M KH_2PO_4 and 0.05 M H_3PO_4 (pH 2.2).
Potential: 20 V/cm.
Detection: U.V. light.
Migration units: cm/h towards anode.

Compound	*Mobility (cm/h)*		
	E_1	E_2	E_3
Guanosine	+ 7.0		
1-Methyl-guanosine	+ 5.0	— 0.4	
6-Hydroxy-2-methylamino-purine riboside	+ 5.9	+ 0.6	— 4.5
2-Dimethylamino-6-hydroxypurine riboside	+ 5.9		— 5.1
Cytidine	+ 5.4		— 8.6

TABLE 20

ELECTROPHORETIC MOBILITIES OF METHYLATED GUANINE NUCLEOTIDES

(J. D. SMITH AND D. B. DUNN, *Biochem. J.*, 72 (1959) 294)

Electrolyte: 0.05 M phosphate buffer (pH 2.1).
Detection: U.V. light.
Migration units: cm/h towards anode.

Compound	*Mobility*
Adenosine-3′-phosphate	1.0
Cytidine-3′-phosphate	1.0
2-Dimethylamino-6-hydroxypurine riboside-2′- and 3′-phosphates	2.6
6-Hydroxy-2-methylamino-purine riboside-2′- and 3′-phosphates	2.9
Guanosine-3′-phosphate	3.4
1-Methylguanosine-2′- and 3′-phosphates	2.9
Uridine-3′-phosphate	7.4

TABLE 21

R_F VALUES OF GUANINE AND XANTHINE

(R. BARCHIELLI, G. BORETTI, A. DI MARCO, P. JULITA, A. MIGLIACCI, A. MINGHETTI AND C. SPALLA, *Biochem. J.*, 74 (1960) 382)

Solvents: S_1 = Butanol–formic acid–water (77:10:13, v/v).
S_2 = Water adjusted to pH 10 with aq. NH_3.
Paper: Not stated.
Detection: U.V. light.

Compound	R_F	
	S_1	S_2
Guanine	0.20	0.42
Xanthine		0.60

TABLE 22

R_G VALUES OF SOME OLIGOSACCHARIDES

(G. AVIGAD, *Biochem. J.*, 73 (1959) 587)

Solvents: S_1 = Butanol–ethanol–water (5:2:2, by vol.).
S_2 = Butanol–pyridine–water (5:3:2, by vol.).
Paper: Whatman No. 1.
Time of run: Descending = 72 h; circular = 8 h.
Detection: *p*-Anisidine·HCl; aniline–diphenylamine–phosphoric acid; and where suitable: urea–phosphoric acid; alkaline aq. triphenyltetrazolium chloride.

Compound	R_G	
	S_1	S_2
Sucrose	0.78	0.71
Maltose	0.64	0.45
Isomaltose	0.54	0.41
Turanose	0.84	0.74
Leucrose	0.68	0.50
Maltulose	0.71	0.58
Isomaltulose	0.68	0.53
1-β-Glucosylfructose	0.66	0.48
Isomaltotriose	0.26	0.18
Maltotriose	0.32	0.23
Isomaltotriulose	0.37	0.26
Maltotriulose	0.42	0.34
1-α-Glucosylfructose*	0.64	0.48

* Suggested identity.

TABLE 23

ELECTROPHORETIC MOBILITIES OF SOME OLIGOSACCHARIDES

(G. AVIGAD, *Biochem. J.*, 73 (1959) 587)

Electrolyte: 0.1 *M* sodium borate buffer, pH 10.
Paper: Whatman No. 3MM.
Potential: 20 V/cm; current: 25 mA.
Time of run: 4 h.
Migration units: *M* glucose* (relative).
Detection: *p*-Anisidine·HCl; aniline–diphenylamine–phosphoric acid; and where necessary: urea–phosphoric acid; alkaline aq. triphenyltetrazolium chloride.

Compound	*M glucose**
Sucrose	0.20
Maltose	0.36
Isomaltose	0.66
Turanose	0.73
Leucrose	0.67
Maltulose	0.63
Isomaltulose	0.59
1-β-Glucosylfructose	0.68
Isomaltotriose	0.81
Maltotriose	0.32
Isomaltotriulose	0.31
Maltotriulose	0.35
1-α-Glucosylfructose**	0.70

* Index of migration in paper electrophoresis (A. B. FOSTER, *J. Chem. Soc.*, (1953) 982).
** Suggested identity.

TABLE 24

ELECTROPHORETIC MOBILITIES OF SOME AMINO-SUGARS

(H. R. PERKINS, *Biochem. J.*, 74 (1960) 182)

Electrolyte: 2 N acetic acid.
Paper: Whatman No. 3.
Potential: 15 V/cm.
Apparatus: Kunkel (1954).
Time of run: 250 min.
Migration units: cm towards cathode.
Detection: p-Dimethylaminobenzaldehyde (without heating); aniline hydrogen phthalate.

Compound	*cm*
Glucosamine hydrochloride	24.2
Glucosaminic acid	10.0
Muramic acid	14.8

TABLE 25

ELECTROPHORETIC MOBILITIES OF SOME N-ACETYL-AMINO-SUGARS

(M. J. CRUMPTON, *Biochem. J.*, 72 (1959) 479)

Electrolyte: pH 10 borate buffer (sodium tetraborate, 23.4 g and N NaOH, 30 ml/l).
Paper: Whatman No. 3.
Potential: 1300 V at 25 mA.
Duration: 2.5 h.
Detection: p-Anisidine·HCl.
Units: R_M = rate of movement relative to glucose.

Compound	R_M
N-Acetyl-D-fucosamine	0.14
N-Acetyl-D-glucosamine	0.23
N-Acetyl-D-xylosamine	
N-Acetyl-D-galactosamine	0.33
N-Acetyl-D-allosamine	0.42
N-Acetyl-D-talosamine	0.60
N-Acetyl-D-gulosamine	0.63
N-Acetyl-D-mannosamine	0.65

TABLE 26

R_F VALUES (RELATIVE) OF 6-O-(N-ACETYLGLUCOSAMINYL)-N-ACETYLMURAMIC ACID

(H. R. PERKINS, *Biochem. J.*, 74 (1960) 182)

Solvents: S_1 = Butan-1-ol-acetic acid–water (63:10:27, by vol., organic phase).
S_2 = Butan-1-ol-pyridine–water (6:4:3 by vol.).
Paper: Whatman No. 3.
Time of run: 20 h for S_2.
Temperature of run: 22° for S_2.
Detection: Aniline phthalate.
$R_A = R_F$ relative to N-acetylglucosamine.

Compound	R_A	
	S_1	S_2
6-O-(N-Acetylglucosaminyl)-N-acetylmuramic acid	0.85	0.85

TABLE 27

PAPER ELECTROPHORESIS OF PHENOLIC COMPOUNDS

(J. Halmekoski, *Ann. Acad. Sci. Fennicae, Ser. A II*, No. 96 (1959))

Electrophoresis at pH 7–8 in buffer solutions (Clark and Lub NaOH–KH_2PO_4 buffers) containing chelate-forming anions, paper Whatman No. 1, room temperature, current 8–10 mA (constant), time 13 h 45 min–20 h 50 min.

Chelating agent added per l of buffer	*pH*	*Time* *h*	*min*	*Const. current mA*	*True migration in cm* *Vanillyl alcohol*	*Acetoguaiacone*	*Vanillin*	*Vanillic acid*	*Catechol*	*3,4-Dihydroxybenzaldehyde*	*3,4-Dihydroxybenzoic acid*	*3,4,5-Trihydroxybenzoic acid*
0.02 mole Na_2MoO_4	7	17	40	10	0.0	1.2	3.2	10.4	14.3	14.0	22.4	20.8
0.02 mole Na_2WO_4	7	20	50	8	0.1	2.9	6.8	11.0	18.4	18.1	24.2	22.7
0.001 mole Na phosphomolybdate	7	15	30	10	0.0	0.7	2.9	12.4	17.0	17.0	24.2	21.2
0.001 mole Na phosphotungstate	7	18	00	8	0.0	2.8	6.0	9.1	15.2	15.2	20.0	18.8
0.02 mole NH_4VO_3	7	15	30	8	0.0	1.9	3.7	10.4	13.6	15.8	20.1	22.7
0.01 mole Na stannate	8	13	45	8	0.1	4.5	8.7	13.2	16.4	16.8	24.9	*
0.02 mole borax**	7.5	17	30	8	0.2	4.6	8.3	13.2	14.9	14.9	24.1	21.1

* Because of irregular and elongated spots difficult to determine center of spot.

** Migration of pyrogallol 12.3 cm.

TABLE 28

R_F VALUES OF PHENOLIC COMPOUNDS

(J. HALMEKOSKI, *Ann. Acad. Sci. Fennicae. Ser. A II*, No. 96 (1959))

Paper or eluents buffered with different anions; paper Whatman No. 1, temperature 18–20°, running time 5–22 hours.

Eluent used	*Saturation*			R_F									
	Compound used	*Added to*		*Vanillyl alcohol*	*o-Vanillin*	*Acetoguaiacone*	*Vanillin*	*Vanillic acid*	*Catechol*	*3,4-Dihydroxybenzaldehyde*	*3,4-Dihydroxybenzoic acid*	*Pyrogallol*	*3,4 5-Trihydroxybenzoic acid*
		eluent	*paper*										
K_2HPO_4–NaOH buffer at pH 7	0.01 mole Na_2MoO_4/l	+		0.77	0.69	0.60	0.64	0.69	0.66	0.83	0.92	—	—
Pyridine–*n*-butanol–H_2O (1:1:2)	Na_2WO_4	+		0.88	0.89	—	0.86	0.66	0.92	0.92	0.66	0.88	0.29
Pyridine–*n*-butanol–H_2O (1:1:2)	Na phosphotungstate	+		0.89	0.92	—	0.92	0.70	0.94	0.96	0.69	0.91	0.64
Pyridine–*n*-butanol–H_2O (1:1:2)	Na_2MoO_4		+	0.96	0.96	—	0.93	0.70	0.77*	0.77*	—	0.96*	0.38*
Pyridine–*n*-butanol–H_2O (1:1:2)	NaCl	+		—	—	—	—	—	0.96	—	0.74	0.94	0.43
Pyridine–*n*-butanol (1:1)	Na_2MoO_4		+	0.91	0.90	—	0.87	0.58	0.73	0.71	0.02	0.90	0.00
Pyridine–*n*-butanol (1:1)	NH_4VO_3		+	0.89	0.87	—	0.92	0.79	0.77	0.78	0.56	0.92	0.36
n-Butanol–acetic acid–H_2O (4:1:5)	Na_2MoO_4		+	0.88	0.97	—	0.96	0.91	0.57	0.18	0.15	0.14	0.05
n-Butanol–acetic acid–H_2O (4:1:5)	Na_2WO_4		+	0.89	0.95	—	0.94	0.89	0.82	0.36	0.32	0.19	0.04
n-Butanol–acetic acid–H_2O (4:1:5)	Na phosphomolybdate		+	0.93	0.97	—	0.96	0.92	0.60	0.23	0.16	0.12	0.03
n-Butanol–acetic acid–H_2O (4:1:5)	Na phosphotungstate		+	0.85	0.92	—	0.91	0.90	0.88	0.82	0.81	0.74	0.61
n-Butanol–acetic acid–H_2O (4:1:5)	Na_2HAsO_3		+	0.95	0.98	—	0.96	0.87	0.93	0.89	0.72	0.80	0.41
n-Butanol–acetic acid–H_2O (4:1:5)	$NaVO_3$		+	0.90	—	0.95	0.94	0.92	*	0.40	0.40	0.16	0.13
n-Butanol–acetic acid–H_2O (4:1:5)	borax		+	0.88	0.95	0.94	0.93	0.88	0.89	0.70	0.64	—	0.31
n-Butanol–acetic acid–H_2O (4:1:5)	Na_2SO_4		+	0.86	0.92	—	0.93	0.91	0.91	0.90	0.87	0.75	0.64
n-Butanol–acetic acid–H_2O (4:1:5)	—			—	—	—	—	0.92	0.91	—	0.85	0.77	—
n-Butanol satd. with H_2O	Na_2MoO_4		+	0.89	—	0.94	0.94	0.29	0.36	0.07	0.00	0.00	0.00
n-Butanol satd. with H_2O	Na_2WO_4		+	0.92	—	0.87	0.72	0.11	0.09	0.05	0.00	0.00	0.00
n-Butanol satd. with H_2O	Na phosphomolybdate		+	0.92	—	0.96	0.94	0.18	0.33	0.09	0.03	0.03	0.03
n-Butanol satd. with H_2O	Na phosphotungstate		+	0.81	—	0.88	0.88	0.81	0.84	0.74**	0.54	0.66**	0.24**
n-Butanol satd. with H_2O	Na_2HAsO_3		+	0.88	—	0.68	0.52	0.05	0.71	0.35**	0.01	0.06	0.00
n-Butanol satd. with H_2O	$NaVO_3$		+	0.88	—	0.93	0.91	0.38	0.43	0.07	0.00	0.02	0.00
n-Butanol satd. with H_2O	borax		+	0.89	—	0.84	0.71	0.10	0.25	0.20	0.00	0.08**	0.00
n-Butanol satd. with H_2O	Na_2SO_4		+	0.84	—	0.88	0.88	0.68	0.88	0.83	0.44	0.72	0.17
n-Butanol satd. with H_2O	Na borate		+	—	—	—	—	—	0.40	0.28	0.02	0.16	—

* Diffuse spots.

** Tailing spots.

TABLE 29

R_F VALUES OF SOME PIPERIDINE-2-CARBOXYLIC ACIDS AND RELATED COMPOUNDS

(M. FERLES AND M. PRYSTAŠ, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 3326)

Solvent: *n*-Butanol–acetic acid–water (4:1:1) (atmosphere: 20% acetic acid and *n*-butanol).
Paper: Whatman No. 1 (descending).
Temperature of run: 16.5°.
Length of run: 20 cm.
Detection: Ninhydrin (secondary amino acids); iodine vapour (tertiary amino acids).

Compound	R_F
Picolinic acid	0.87
Pipecolinic acid	0.82
Baikiain	0.89
N-Methylbaikiain	0.87
N-Methylpipecolinic acid	0.85

TABLE 30

R_F VALUES OF SOME SULPHOLANES AND RELATED COMPOUNDS

(M. PROCHÁZKA AND V. HORÁK, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 2278)

Solvent: *n*-Butanol–acetic acid–water (4:1:5, organic phase).
Paper: Whatman No. 4.

Compound	R_F
3-Hydroxy-4-aminosulpholane	0.27–0.30
4-Hydroxy-2-sulpholene	0.63–0.67
3-Hydroxy-4-aminosulpholane hydrochloride	0.13
3a,7a-Dihydrobenzothiophen 1,1-dioxide	0.86
4-Amino-2-sulpholene	0.34–0.37
4-Amino-2-sulpholene dimer	0.03
3,4-Diaminosulpholane	0.26–0.29
Bis-(4-amino-3-sulpholanyl)-amine*	0.12
3,4-Diaminosulpholane hydrochloride	0.05

* Tentative identification.

TABLE 31

R_F VALUES OF SOME BRUCINES AND RELATED COMPOUNDS

(Z. DEYL AND M. EFFENBERGER, *Collection Czechoslov. Chem. Communs.*, 24 (1959) 3763)

Solvent: *n*-Butanol–butyl acetate–acetic acid–water (1:1:2:1).
Paper: Whatman No. 2 (descending).
Detection: 1% ethanolic *p*-dimethylaminobenzaldehyde after $ZnCl_2$ reduction; 15% ferric chloride and 1% potassium ferricyanide (1:1); Pauly; (depending on the compound).

Compound	R_F
Bisdemethylbrucine	0.47
Nitrobisdemethylbrucine	0.61
Dinitrostrychnolcarboxylic acid	0.49
3,5-Dinitrobenzoic acid	0.89
2,4,6-Trinitrophenol	0.94

TABLE 32

R_F VALUES OF ALKALI METALS

(E. C. MARTIN, *Anal. Chim. Acta*, 22 (1960) 142)

Metal ion	R_F	
	Methanol containing 1% 15N ammonia	*Methanol containing 5% 15 N ammonia*
Li	0.82	0.74
Na	0.40	0.30
K	0.19	0.12
Rb	0.16	0.12
Cs	0.16	0.10
Mg	0	0

TABLE 33

R_F VALUES OF Mn, Co AND Cu IN TRIBUTYL PHOSPHATE–HCl–METHANOL MIXTURES

(G. WEIDMANN, *Can. J. Chem.*, 38 (1960) 459)

Paper: Schleicher and Schüll 4023b.
Temperature: 22°.
Solvent: 200 ml portions of purified tributyl phosphate (TBP) are shaken repeatedly with the required concentration of aqueous HCl till the concentration of the aqueous phase remains constant. Two parts of this TBP–HCl mixture are then mixed with one part of methanol.

TBP adjusted with HCl of molarity	R_F		
	Mn	*Co*	*Cu*
5.5	0.15	0.16	0.42
6.5	0.20	0.23	0.48
7.5	0.24	0.28	0.52
8.5	0.30	0.35	0.54
9.0	0.31	0.38	0.56
9.5	0.34	0.43	0.57
10.0	0.35	0.46	0.57
10.5	0.36	0.48	0.57
11.0	0.38	0.51	0.58
11.5	0.42	0.61	0.59

TABLE 34

R_F VALUES OF INORGANIC ANIONS

(H. LAUB, *Z. anal. Chem.*, 173 (1960) 208)

Solvent: Butanol–acetone–water (5:2:3).
Paper: Schleicher & Schüll 2040b.

Anion	R_F	*Anion*	R_F
ClO_4^-	0.67	Cl^-	0.36
ClO_3^-	0.50	NO_3^-	0.46
ClO_2^-	0.38	NO_2^-	0.41
I^-	0.55	BrO_3^-	0.34
Br^-	0.43	IO_3^-	0.19

TABLE 35

R_F VALUES OF SOME URIDINE PHOSPHATES

(J. SMRT AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 553)

Solvents: S_1 = *n*-Butanol–acetic acid–water (5:2:3).
S_2 = Isopropanol–1 % aqueous ammonium sulphate solution (2:1).
Paper: Whatman No. 1.
Detection: U.V. light; ammonium molybdate–perchloric acid spray.

Uridine derivative	R_F	
	S_1	S_2
Deoxyuridine-5′-phosphate	0.23	0.64
5-Bromodeoxyuridine-5′-phosphate	0.28	0.67
5-Bromouridine-5′phosphate	0.22	0.65

TABLE 36

ELECTROPHORETIC MOBILITIES OF SOME URIDINE DERIVATIVES

(J. SMRT AND F. ŠORM, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 553)

Electrolyte: citrate buffer (pH 3.7).
Paper: Whatman No. 3.
Potential: 100 V/cm.
Apparatus: Markham.
Units: cm/2 h.
Detection: U.V. light; ammonium molybdate–perchloric acid spray.

Uridine derivative	*Mobility*
Deoxyuridine-5′-phosphate	21.8
5-Bromodeoxyuridine-5′-phosphate	21.1
5-Bromouridine-5′-phosphate	21.1

TABLE 37

R_F VALUES OF SOME AMINES AND CHOLINE

(V. KWASNIEWSKI, *Planta Med.*, 7 (1959) 35)

Solvents: S_1 = Butanol–acetic acid–water (4:1:5).
S_2 = Methanol–water (9.5:0.5).
Paper: Schleicher & Schüll 2043b mgl (ascending).
Detection: 0.2 % ninhydrin in 95 % butanol with 5 % 2 *N* acetic acid (amines); 1 % potassium ferrocyanide then 0.5 % cobalt chloride (choline).

Compound	R_F	
	S_1	S_2
Histamine	0.11	0.43
Tyramine	0.44	0.64
Methylamine	0.24	0.10
Choline	0.29	0.62

TABLE 38

R_F VALUES OF N-ACETYL-S-(2-CHLORO-4-NITROPHENYL)-L-CYSTEINE

(H. G. BRAY AND S. P. JAMES, *Biochem. J.*, 74 (1960) 394)

Solvents: S_1 = Butan-1-ol–acetic acid–butyl acetate–water (24:10:5:2, v/v).
S_2 = Butan-1-ol saturated with aqueous 2 *N* NH_3 soln.
Paper: Whatman No. 4 (descending).
Detection: Potassium dichromate–silver nitrate solution; ninhydrin.

Compound	R_F	
	S_1*	S_2**
N-Acetyl-S-(2-chloro-4-nitrophenyl)-L-cysteine	0.89	0.46

* 7 h run.
** 16 h run.

TABLE 39

R_F VALUES OF ARABINOSE AND LYXOSE

(K. G. RIENITS, *Biochem. J.*, 74 (1960) 27)

Solvent: Pyridine–amyl alcohol–water (7:7:6, by vol.).
Paper: Whatman No. 54 (ascending).
Temperature of run: 18°.
Detection: Aniline hydrogen phthalate.

Sugar	R_F
Arabinose	0.47
Lyxose	0.67
(Hexosamine range)	(0.20–0.23)
(Hexuronic acids)	(0.08–0.1)

TABLE 40

R_F VALUES (RELATIVE) OF SOME O-METHYL ETHERS OF RHAMNOSE AND FUCOSE

(A. P. MCLENNAN, D. W. SMITH AND H. M. RANDALL, *Biochem. J.*, 74 (1960) 3P)

Solvent: Butanol–pyridine–water (6:4:3, by vol.).
Detection: Reducing sugar tests; 6-deoxyhexose test (DISCHE AND SHETTLES, 1948).

Compound	$R_{rhamnose}$
Rhamnose	1.00
2-O-Methylfucose	1.18
2-O-Methylrhamnose	1.28
2,4-Di-O-methylrhamnose	1.45

TABLE 41

R_F VALUES OF SOME ANTHOCYANINS

(J. B. HARBORNE, *Biochem. J.*, 74 (1960) 262)

Solvent: Butan-1-ol–2 *N* HCl (1:1, v/v; upper phase).
Paper: Whatman No. 3.
Detection: Visible and U.V. light.

Compound	R_F	*Colour*	
		Visible	*Ultraviolet*
Pelargonidin*	0.44	Orange-red	Fluorescent orange
Cyanidin*	0.35	Magenta	Bright orange
Peonidin*	0.37	Magenta	Fluorescent pink
Delphinidin*	0.30	Mauve	Dull mauve
Petunidin*	0.30	Purple	Dull purple
Malvidin*	0.33	Purple	Fluorescent cerise
Pelargonidin**	0.31	Red	Dull red
Cyanidin**	0.25	Magenta	Dull magenta
Delphinidin**	0.19	Purple	Dull purple
Petunidin**	0.21	Purple	Dull purple

* *p*-Coumaryl 5-glucoside 3-rhamnosylglucoside derivative.
** 3-Rhamnosylglucoside derivative.

TABLE 42

R_F VALUES OF ANTHOCYANIN INTERMEDIATE GLYCOSIDES

(J. B. HARBORNE, *Biochem. J.*, 74 (1960) 262)

Solvents: S_1 = Butan-1-ol–acetic acid–water (4:1:5, by vol.; upper phase).
S_2 = Water–conc. HCl (97:3, v/v).
Paper: Whatman No. 1.
Detection: Visible and U.V. light.

Compound	R_F	
	S_1	S_2
Pelargonidin 5-glucoside	0.51	0.18
Cyanidin 5-glucoside	0.44	0.07
Peonidin 5-glucoside	0.45	0.08
Petunidin 5-glucoside	0.35	0.03
Malvidin 5-glucoside	0.43	0.04
Peonidin 3-rhamnosylglucoside	0.38	0.21
Malvidin 3-rhamnosylglucoside	0.35	0.15

TABLE 43

R_F VALUES OF SOME COUMARINS AND RELATED COMPOUNDS

(J. B. HARBORNE, *Biochem. J.*, 74 (1960) 270)

Solvents: S_1 = Water.
S_2 = Butan-1-ol–acetic acid–water (4:1:5, by vol.).
S_3 = Butan-1-ol–aq. 2 *N* ammonia soln. (1:1, v/v).
S_4 = Aq. 15% acetic acid.
S_5 = Butan-1-ol–ethanol–water (4:1:5, by vol.).
Paper: Whatman No. 3.
Detection: U.V. light (before and after NH_3 treatment).

Compound	*R_F*					*Colour in U.V.*	
	S_1	*S_2*	*S_3*	*S_4*	*S_5*	*Untreated*	*NH_3-treated*
Chlorogenic acid	0.67	0.56	0.00	0.66	0.34	Blue	Green
Scopolin	0.64	0.53	0.44	0.85	0.47	Violet-blue	Violet-blue
Cichoriin	0.61	0.53	0.10	0.78	0.49	Pale pink	Orange-yellow
Aesculin	0.56	0.53	0.13	0.76	0.51	Light blue	Bright blue
Rutin	0.24	0.43	0.23	0.56	0.45	Ochre	Yellow-brown
Isoquercitrin	0.07	0.61	0.23	0.41	0.58	Ochre	Yellow-brown
Fabiatrin	0.79	0.45	0.23	0.85	0.42	Violet-blue	Violet-blue
Caffeic acid	0.25	0.81	0.00	0.56	0.67	Blue	Light blue
Scopoletin	0.29	0.82	0.35	0.59	0.80	Violet-blue	Bright blue
Aesculetin	0.28	0.75	0.13	0.56	0.68	Blue	Light blue
Quercetin	0.00	0.71	0.00	0.05	0.70	Bright yellow	Yellow
6-Hydroxy-7-methoxycoumarin	0.21	0.83	0.54	0.51	0.81	Pale pink	Orange-yellow

TABLE 44

R_F VALUES OF SOME CONDENSED TANNINS

(D. G. ROUX AND E. A. MAIHS, *Biochem. J.*, 74 (1960) 44)

Solvents: S_1 = 2% acetic acid (ascending).
S_2 = Butan-1-ol–acetic acid–water (6:1:2).
S_3 = Butan-1-ol–acetic acid–water (4:1:5).
S_4 = Butan-2-ol saturated with water.
S_5 = Water.
Paper: Whatman No. 3 (18¼ in. × 22½ in.).
Detection: U.V. light; vanillin–toluene-*p*-sulphonic acid; bis-diazotized benzidine; $FeCl_3$ solution.

Compound	*R_F*				
	S_1	*S_2*	*S_3*	*S_4*	*S_5*
(—)-7,3′,4′,5′-Tetrahydroxyflavan-3-ol [(—)-robinetinidol]	0.47	—	0.76	0.67	—
(+)-Catechin			0.75	0.67	0.35
(+)-Gallocatechin			0.57	0.54	0.32

TABLE 45

R_F VALUES OF SOME FLAVONOLS

(V. KWASNIEWSKI, *Planta Med.*, 7 (1959) 35)

Solvents: S_1 = Butanol–acetic acid–water (4:1:5).
S_2 = Pyridine–ethyl acetate–water (1:2:2).
S_3 = Butanol–acetone–water (4:1:5).
S_4 = Ethyl acetate–formic acid–water (10:2:3).
S_5 = Benzene–acetic acid–water (4:4:2).
Paper: Schleicher & Schüll 2043b mgl.
Detection: Zirconium–citric acid (HÖRHAMMER, 1954); Zn/HCl; Mg/HCl (flavonols); aniline phthalate (glucose).

Compound	R_F				
	S_1	S_2	S_3	S_4	S_5
Quercetin-3-glucoside	0.36		0.34		0.54
Kaempferol-3-glucoside	0.85		0.85		0.84
Quercetin	0.68		0.74		0.45
Kaempferol	0.88		0.89		0.83
Glucose	0.19	0.22	0.19	0.09	0.82

TABLE 46

R_F VALUES OF VERBASCOSIDE AND ITS AGLYCONE

(S. HEIN, *Planta Med.*, 7 (1959) 185)

Solvent: S_1 = Butanol–acetic acid–water (4:1:5).
S_2 = Ethyl acetate–formic acid–water (10:2:3).
Paper: P_1 = Schleicher & Schüll 2043b.
P_2 = Schleicher & Schüll 2045b.
Detection: U.V. light; $AlCl_3$ solution; magnesium acetate solution (flavonoids); aniline phthalate (sugars).

Compound	R_F			
	S_1		S_2	
	P_1	P_2	P_1	P_2
Verbascoside*	0.35	0.28	0.36	0.26
Verbascoside aglycone	0.78	0.76	0.86	0.98
Glucose	0.18			
Rhamnose	0.35			

* A flavonol-3-glycoside from *Verbascum phlomoides*.

TABLE 47

R_F VALUES OF SOME IODINE COMPOUNDS

(C. CAMERON, *Biochem. J.*, 74 (1960) 333)

Solvents: S_1 = Butanol–2 *N* acetic acid (1:1, v/v).
S_2 = Butanol–aq. 2 *N* NH_3 soln. (1:1, v/v).
S_3 = Butanol–aq. 6 *N* NH_3 soln. (1:1, v/v).
S_4 = Butanol–dioxan–aq. 2 *N* NH_3 soln. (4:1:5, by vol.).
S_5 = Butanol–pentanol–aq. 2 *N* NH_3 soln. (4:1:5, by vol.).
S_6 = *tert.*-Pentanol–aq. 2 *N* NH_3 soln. (1:1, v/v).
Paper: Whatman No. 3 MM (descending).
Detection: Ilford Industrial X-ray film, Mica end-window Geiger tube (EW3H 20th Century Electronics Ltd.); ceric sulphate–sodium arsenite (1% and 5% (w/v) resp. in *N* H_2SO_4 mixed immediately before use), then 1% (w/v) *o*-phenylenediamine in acetone.

Compound	R_F					
	S_1	S_2	S_3	S_4	S_5	S_6
Iodide	0.15	0.29	0.35	0.41	0.16	0.20
Monoiodotyrosine	0.54	0.17	—	0.28	0.05	0.14
3,5-Diiodotyrosine	0.84	0.05	—	0.16	—	—
3,5-Diiodo-4-hydroxyphenyllactic acid	0.95	0.08	—	0.20	0.03	0.06
3,5-Diiodo-4-hydroxyphenylpyruvic acid	0.90	—	—	0.82	—	—
3-Monoiodothyronine	—	—	0.55	0.79	0.69	—
3,5-Diiodothyronine	—	0.79	0.85	0.85	0.68	0.90
3,3′-Diiodothyronine	—	—	0.45	0.60	0.69	0.75
3,5,3′-Triiodothyronine	—	0.75	0.81	0.78	0.73	0.66
3,3′,5′-Triiodothyronine	—	—	0.45	0.53	0.60	0.45
Thyroxine	—	0.62	0.72	0.61	0.64	0.45
Tetraiodothyroacetic acid	—	0.78	—	0.82	0.80	0.72
Triiodothyroacetic acid	—	0.69	—	0.69	0.65	0.84

TABLE 48

R_F VALUES OF BERBERINE AND OTHER *Chelidonium majus* ALKALOIDS

(F. KACZMAREK AND B. MALEK, *Planta Med.*, 7 (1959) 171)

Solvent: Carbon tetrachloride–chloroform–benzene (proportions not stated).
Paper: Schleicher & Schüll 2043b mgl (ascending).
Impregnation: Formamide–benzoic acid–acetone (6 ml:0.4 g:18 ml); acetone allowed to evaporate (24 h).
Length of run: 20 cm.
Time of run: 1 h.
Detection: Modified Dragendorff (AMELINK, 1934); U.V. light.

Compound	R_F
Chelidonine	0.83
Sanguinarine	0.62
Chelerythrine	0.44
Protopine	0.18
β-Allocryptopine	0.06
Berberine	0.00

TABLE 49

R_F VALUES OF SOME *Senecio* ALKALOIDS

(H.-B. SCHRÖTER AND F. ŠANTAVÝ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 472)

Solvents: S_1 = *n*-Butanol–acetic acid (5%)[a,b].
S_2 = Isobutanol–toluene–water (1:1, satd. 5% for 48 h)[c].
S_3 = *n*-Propanol–water (3:1) (paper impregnated with pH 5.5 phosphate buffer and dried at 80°).
S_4 = *n*-Butanol–water (satd.) (paper impregnated with borate buffer pH 7.8).
Paper: P_1 = Whatman No. 1 (used by authors).
P_2 = Schleicher & Schüll 2043b (12 cm diam.) for circular paper chromatography.
Detection: Dragendorff (dip).
(*a*) = ascending; (*d*) = descending.

Alkaloid	*Formula*	R_F				
		S_1		S_2[c] P_1 (*d*)	S_3 P_1 (*a*)	S_4 P_2
		(*a*)	(*d*)			
Integerrimine	$C_{18}H_{25}NO_5$		0.61[a]			
Senecionine	$C_{18}H_{25}NO_5$	0.56	0.64 , 0.62[a]	0.56	0.58	0.80
Seneciphylline	$C_{18}H_{23}NO_5$	0.52	0.52[b], 0.58[a]			
Retrorsine	$C_{18}H_{25}NO_5$		0.44[a]			
Riddelliine	$C_{18}H_{23}NO_6$	0.35	0.40[a]		0.37	0.69
Trichodesmine	$C_{18}H_{27}NO_6$		0.54[a]			
Junceine	$C_{18}H_{27}NO_7$		0.38[a]			
Jaconine	$C_{18}H_{23}NO_5$		0.43[b]			
Jacoline	$C_{18}H_{27}NO_7$		0.26[a,b]			
Jacozine	$C_{18}H_{23}NO_6$		0.32[b]			
Jacobine	$C_{18}H_{25}NO_6$		0.39[b], 0.44[a]			
Tomentosine	$C_{19}H_{27}NO_7$		0.40[d]			
Otosenine	$C_{19}H_{27}NO_7$	0.39	0.41	0.39	0.47	0.54
Alkaloid S-C	$C_{18}H_{23}NO_6$	0.36	0.38	0.36	0.41[e]	0.76
Alkaloid S-D	$C_{18}H_{25}NO_5$	0.56	0.64	0.59	0.58[e]	0.90
Senecic acid	$C_{10}H_{16}O_5$	0.92	0.90[f]			
Jaconecic acid	$C_{10}H_{16}O_6$	0.47	0.52[f]			

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[a] R. ADAMS AND M. GIANTURCO, *J. Am. Chem. Soc.*, 78 (1956) 398, 1919, 1926.
[b] R. B. BRADBURY AND C. C. J. CULVENOR, *Australian J. Chem.*, 7 (1954) 378.
[c] J. BÜCHI AND H. SCHUMACHER, *Pharm. Acta Helv.*, 31 (1956) 417.
[d] R. ADAMS, M. GIANTURCO AND B. L. VAN DUREN, *J. Am. Chem. Soc.*, 78 (1956) 3513.
[e] Appear as brown spots first.
[f] R. B. BRADBURY AND J. B. WILLIS, *Australian J. Chem.*, 9 (1956) 258.

TABLE 50

R_F VALUES OF SOME POLYHYDRIC ALCOHOLS

(J. BORECKÝ AND J. GASPARIČ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1287)

Solvents: S_1 = Water-saturated ethyl acetate.
S_2 = Ethyl acetate–ethanol–water (12:2:1).
Paper: Whatman No. 3 (descending).
Detection: 5 % aq. $AgNO_3$ (9 ml) + ammonia (1 ml) spray, then heated to 100° (15–40 min), fixation by water wash (15 min), then fixation with 10 % thiourea in 0.1 *N* H_2SO_4; saturated aq. potassium periodate spray; 1 *N* NaOH–2 % triphenyl-tetrazolium chloride (1:1) spray, then heated to 100° for a short time.

Alcohol	R_F	
	S_1	S_2
Glucose	0	0
d-Arabitol	0	0.05
Glycerol	0.02	0.21
Ethylene glycol	0.15	0.43
1,2-Propylene glycol	0.35	0.58
1,4-Butylene glycol	0.32	0.62
1,3-Butylene glycol	0.42	0.67
2,3-Butylene glycol	0.55	0.70

TABLE 51

R_F VALUES OF 3,5-DINITROBENZOATES OF SOME ALCOHOLS AND THEIR DERIVATIVES

(J. BORECKÝ AND J. GASPARIČ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1287)

Solvent: Hexane.
Paper: Whatman No. 3 (descending).
Impregnation: I_1 = 50 % dimethyl formamide.
I_2 = 25 % dimethyl formamide.
I_3 = 20 % formamide
(Care must in be taken in removal of formamides.)
Detection: Unfiltered U.V. irradiation; Zn(II) chloride reduction, then *p*-dimethylaminobenzaldehyde spray.

3,5-Dinitrobenzoate of	R_F		
	I_1	I_2	I_3
Ethylene glycol monomethyl ether	0.04	0.24	0.20
Ethylene glycol monoethyl ether	0.10	0.46	0.43
Ethylene glycol mono-*n*-butyl ether	0.27	0.71	0.76
Diethylene glycol mono-*n*-butyl ether	0.20	0.63	0.59
Methanol	0.08	0.34	0.31
Ethanol	0.16	0.57	0.59
n-Propanol	0.29	0.70	0.76
n-Butanol	0.41	0.70	0.86
n-Amyl alcohol	0.53	0.84	—
α-Dichlorohydrin	0.06	0.31	0.42
Ethylene chlorohydrin	0.04	0.22	0.26
1,2-Propylene chlorohydrin	0.08	0.45	0.58

TABLE 52

R_F VALUES OF 3,5-DINITROBENZOATES OF HYDROXYETHYLATED *n*-BUTANOL, OTHER ALIPHATIC ALCOHOLS AND RELATED COMPOUNDS

(J. BORECKÝ AND J. GASPARIČ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1287)

Solvent: S_1 = Hexane.
S_2 = Chloroform–ethyl acetate (2:8).
Impregnation: 20 % formamide.
Detection: Unfiltered U.V. irradiation: Zn(II) chloride reduction, then *p*-dimethylaminobenzaldehyde spray.

3,5-Dinitrobenzoate of	R_F	
	S_1	S_2
Methanol	0.22	—
Ethanol	0.47	—
n-Propanol	0.65	—
n-Butanol	0.78	—
n-Amyl alcohol	0.86	—
Ethylene glycol mono-*n*-butyl ether	0.67	—
Diethylene glycol mono-*n*-butyl ether	0.51	—
Triethylene glycol mono-*n*-butyl ether	0.31	—
Tetraethylene glycol mono-*n*-butyl ether	0.16	—
Glycerol triester	—	0.93
Glycerol monoester	—	0.48
3,5-Dinitrobenzoic acid	—	0.04

TABLE 53

R_F VALUES OF 3,5-DINITROBENZOATES OF GLYCOLS

(J. BORECKÝ AND J. GASPARIČ, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1287)

Solvent: S_1 = Hexane–benzene (3:1).
S_2 = Hexane–benzene (1:3).
Paper: Whatman No. 3 (descending).
Impregnation: 20 % formamide.
Detection: Unfiltered U.V. irradiation; Zn(II) chloride reduction then *p*-dimethylaminobenzaldehyde spray.

3,5-Dinitrobenzoate of	R_F	
	S_1	S_2
1,3-Butylene glycol diester	0.48	0.92
1,2-Propylene glycol diester	0.42	—
Ethylene glycol diester	0.21	0.86
Ethylene glycol monoester	at start	0.12
1,2-Propylene glycol monoester	0.02	0.28
1,3-Butylene glycol monoester	0.06	0.50
Methanol	0.66	—
Ethanol	0.83	—
Glycerol triester	—	0.72
Glycerol monoester	—	at start

TABLE 54

R_F VALUES OF AMINOACETONE AND δ-AMINOLAEVULINIC ACID

(W. H. ELLIOTT, *Biochem. J.*, 74 (1960) 478)

Solvents: S_1 = 2-Methylpropan-2-ol–methyl ethyl ketone–formic acid–water (120:50:40:40).
S_2 = Ethanol–*M* acetic acid–pyridine–water (95:10:3:3).
Paper: Whatman No. 3 (washed by allowing distilled water to drip off bottom of sheets for three days).
Detection: Ninhydrin; copper–ninhydrin [satd. $Cu(NO_3)_2$ in acetone (0.4 ml/100 ml) dip; 0.2% ninhydrin in acetone dip]; picrate spray (aq. satd. picric acid–10 *N* NaOH–water, 10:20:50), then sprayed with 5 *N* HCl after 1 min; modified Morgan-Elson [*M* acetate (pH 4.6) buffer–acetylacetone (10:1); autoclave paper 3 min at 15 lb./in.^{2} pressure, then spray with modified Ehrlich of MAUZERALL AND GRANICK (1956)].

Compound	R_F	
	S_1	S_2
Aminoacetone	0.4	0.3
δ-Aminolaevulinic acid	0.4	0.15

TABLE 55

R_F VALUES OF SOME DINITROPHENYL-AMINO ACIDS

(J. C. CRAWHALL AND R. W. E. WATTS, *Biochem. J.*, 74 (1960) 514)

Solvent: 2-Methylbutan-2-ol system (BLACKBURN AND LOWTHER, 1951).
Paper: Whatman No. 1 (buffered).
Detection: Visible light.

Compound	R_F
DNP-glycine	0.38
DNP-δ-aminolaevulinic acid	0.63

TABLE 56

R_F VALUES OF PHOSPHORYLCHOLINE AND PHOSPHORYLETHANOLAMINE

(M. W. NEIL, *Biochem. J.*, 74 (1960) 32P)

Solvent: Phenol–ammonia.
Paper: Not stated (presumably Whatman).
Detection: Phosphate reagent.

Compound	R_F
Phosphorylcholine	0.85
Phosphorylethanolamine	0.35

TABLE 57

R_F VALUES OF SOME PHOSPHOLIPID HYDROLYSIS PRODUCTS

(R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 45)

Solvents: S_1 = Phenol saturated with water–acetic acid–ethanol (100:10:12, by vol.), freshly prepared.

S_2 = Methanol–98% formic acid–water (80:13:7, by vol.), freshly prepared.

Paper: Whatman No. 1, washed with 2 *N* acetic acid then water (ascending: S_1 and S_2; descending S_1).

Time of run: 14–18 h (S_1).

8 h (S_2).

Detection: 0.25% ninhydrin in acetone (3 min at 100°); acid molybdate spray followed by U.V. irradiation; perchloric acid–hydrochloric acid acidified molybdate followed by U.V. irradiation.

Hydrolysis product	R_F*	
	S_1	S_2
Glycerylphosphorylcholine	0.77	0.67
Glycerylphosphorylethanolamine	0.63	0.47
Glycerylphosphorylserine	0.30	0.50
Glycerylphosphorylinositol	0.20	0.45
Phosphorylinositol	0.20	0.45
Glycerophosphoric acid	0.33	0.74
Polyglycerol phosphate	0.28	0.60
Cyclic glycerophosphoric acid	0.50	0.66
Inorganic P	0.25	0.85

* Approximate.

TABLE 58

ELECTROPHORETIC MOBILITIES OF SOME PHOSPHOLIPID HYDROLYSIS PRODUCTS

(R. M. C. DAWSON, *Biochem. J.*, 75 (1960) 45)

Electrolyte: E_1 = Pyridine–acetic acid–water (1:10:89, by vol.), a volatile buffer at pH 3.6.

Paper: Whatman No. 1 washed with 2 *N* acetic acid then water.

Potential: 2000V.

Time of run: 55 min.

Apparatus: RYLE, SANGER, SMITH AND KITAI (1955), paper under water-cooled toluene.

Mobility units: Relative to inorganic P (based on cm towards anode).

Detection: 0.25% ninhydrin in acetone (3 min at 100°); acid molybdate spray followed by U.V. irradiation; perchloric acid–hydrochloric acid acidified molybdate followed by U.V. irradiation.

Hydrolysis product	*Mobility*
Glycerylphosphorylcholine	0
Glycerylphosphorylethanolamine	0
Glycerylphosphorylserine	0.61
Glycerylphosphorylinositol	0.57
Phosphorylinositol	0.64
Glycerophosphoric acid	0.82
Polyglycerolphosphate	0.96
Cyclic glycerophosphoric acid	1.0
Inorganic P	1.0

TABLE 59

R_F VALUES OF TWO XANTHURENIC ACID CONJUGATES

(C. BAGLIONI, P. FASELLA, C. TURANO AND N. SILIPRANDI, *Biochem. J.*, 74 (1960) 521)

Solvents: S_1 = Butanol–acetic acid–water (4:1:5, by vol.).
S_2 = 20% (w/v) KCl.
Paper: Not stated.
Detection: Ninhydrin, Pauly reagent, U.V. light.

Compound	R_F	
	S_1	S_2
Xanthurenic acid conjugate I*	0.14	0.47
Xanthurenic acid conjugate II**	0.09	0.53

* Tentatively identified as having 8-OH bound to sulphate and carboxyl group to glycine.
** Tentatively identified as having 8-OH bound to glucuronic acid and carboxyl group to glycine.

TABLE 60

ELECTROPHORETIC MOBILITIES OF TWO XANTHURENIC ACID CONJUGATES

(C. BAGLIONI, P. FASELLA, C. TURANO AND N. SILIPRANDI, *Biochem. J.*, 74 (1960) 521)

Electrolyte: Potassium phosphate buffer (pH 5.9, *I* 0.05).
Paper: Not stated.
Potential gradient: 10 V/cm.
Method: Horizontal.
Units: cm (anodic displacement).
Time of run: 8 h.
Detection: Ninhydrin, Pauly reagent, U.V. light.

Compound	*Mobility*
Xanthurenic acid conjugate I*	25.00
Xanthurenic acid conjugate II**	14.50

* Tentatively identified as having 8-OH bound to sulphate and carboxyl group to glycine.
** Tentatively identified as having 8-OH bound to glucuronic acid and carboxyl group to glycine.

TABLE 61

R_F VALUE OF 5-HYDROXYTRYPTACYAMINE CREATINE SULPHATE

(D. B. HOPE AND A. D. SMITH, *Biochem. J.*, 74 (1960) 101)

Solvent: Butan-1-ol–acetic acid–water (4:1:5, upper phase).
Paper: Whatman No. 4.
Detection: Spray of: (A) diacetyl (1%, v/v, in water; 2.5 ml) with α-naphthol (25%, w/v, in propan-1-ol; 20 ml) made up to 100 ml with propan-1-ol. (B) 3 *N* NaOH. Immediately before use, 10 ml of (A) and 5 ml of (B) made up to 30 ml with water.

Compound	R_F
5-Hydroxytryptacyamine creatine sulphate	0.48

TABLE 62

R_F VALUES OF SOME ANTHRAQUINONE DERIVATIVES

(J. FRANC AND M. WURST, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 657)

Solvent: 80% ethanol.
Paper: Whatman No. 1, impregnated with 30% liquid paraffin (b.p. 190–275°) in benzene; descending.
Detection: U.V. light; visible light.
Standard: 1-Anilinoanthraquinone used as control on all chromatograms.

Anthraquinone	R_F	*Anthraquinone*	R_F
1-Anilino-	0.28	1-Amino-2-bromo-4-hydroxy-	0.15
1-Hydroxy-	0.42	1-Sulpho- (sodium salt)	0.49
2-Hydroxy-	0.70	2-Sulpho- (sodium salt)	0.58
1,2-Dihydroxy-	0.71	1,6-Disulpho- (sodium salt)	0.11
1,4-Dihydroxy-	0.34	1,7-Disulpho- (sodium salt)	0.18
1,5-Dihydroxy-	0.26	1,8-Disulpho- (sodium salt)	0.17
1,6-Dihydroxy-	0.85	2,6-Disulpho- (sodium salt)	0.22
1,8-Dihydroxy-	0.37	2,7-Disulpho- (sodium salt)	0.27
2,6-Dihydroxy-	0.85	1-Chloro-2-carboxy-	0.57
2,7-Dihydroxy-	0.90	2-Carboxy-	0.50
1,2,5,8-Tetrahydroxy-	—	1-Nitro-	0.59
1,4,5,8-Tetrahydroxy-	0.00	4-Nitro-1,8-dichloro-	0.63
1-Anilino-	0.28	1-Nitro-2-methyl-	0.62
1-Amino-	0.74	1-Nitro-5-sulpho-	0.50
2-Amino-	*	1-Nitro-6-sulpho-	0.58
1,5-Diamino-	0.70	1-Nitro-8-sulpho-	0.48
1,8-Diamino-	0.68	1-Nitro-7-sulpho-	0.58
2,3-Diamino-	0.72	1-Nitro-2-carboxy-	0.58
1,2-Diamino-	0.71	1-Nitro-5-chloro-	0.52
1-Amino-2-hydroxy-	0.71	1-Nitro-2-carbonylchloride	0.57
1-Amino-4-hydroxy-	0.70	2-Sulphochloride	0.66
2-Amino-3-hydroxy-	0.53	1,5-Disulphochloride	0.18
1-Amino-4-hydroxy-5-chloro-	0.65	1,8-Disulphochloride	0.25
2-Amino-1,3-dibromo-	0.65	2,6-Disulphochloride	0.41
1-Amino-2-hydroxyethoxy-4-hydroxy-	0.58	2,7-Disulphochloride	0.41
1,5-Diamino-2,4,6,8-tetrabromo-	0.34	1-Bromo-	0.46
1-Amino-2-sulpho-	0.63	2-Bromo-	0.33
1-Amino-2-carbonylchloride	0.52	1,3-Dibromo-	0.09
1-Amino-2-sulpho-4-bromo-	0.54	1,5-Dibromo-	—
1,5-Diamino-2-sulpho-	0.45	1,3,5,7-Tetrabromo-	0.03
1-Amino-2-chloro-	0.34	Anthraquinone	0.45
1-Amino-2-sulpho-4-bromo-	0.45 *	2-Methyl-	0.46
2-Amino-3-chloro-	0.52	Dianthrimide	0.00
1-Amino-2,4-dibromo-	0.15 *	4,4′-Diaminodianthrimide	0.00

* Elongated spots.

TABLE 63

ELECTROPHORETIC MOBILITIES OF SOME ANTHRAQUINONE DERIVATIVES

(J. FRANC AND M. WURST, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 657)

Electrolytes: $E_1 = 3\ N\ NH_4OH$.
$E_2 = 1\ N\ CH_3COOH$.
Paper: Whatman No. 1.
Mobility units: $u \times 10^5\ cm^2 \cdot V^{-1} \cdot sec^{-1}$.
Potential: 6–7 V/cm.
Detection: U.V. light; visible light.
Standard: 3-Nitrophthalic acid ($u = 20.5$).

Anthraquinone	*Mobility*	
	E_1	E_2
1-Anilino-		0.0
1-Hydroxy-	0.0	0.0
2-Hydroxy-	4.6	0.0
1,2-Dihydroxy-	0.0	0.0
1,4-Dihydroxy-	0.0	0.0
1,5-Dihydroxy-	0.0	0.0
1,6-Dihydroxy-	1.75	0.0
1,8-Dihydroxy-	0.0	0.0
2,6-Dihydroxy-	6.6	0.0
2,7-Dihydroxy-	7.7	0.0
1,2,5,8-Tetrahydroxy-	0.0	0.0
1,4,5,8-Tetrahydroxy-	0.0	0.0
1-Amino-	0.0	0.0
2-Amino-	0.0	0.0
1,5-Diamino-	0.0	0.0
1,8-Diamino-	0.0	0.0
2,3-Diamino-	0.0	0.0
1,2-Diamino-	0.0	0.0
1-Amino-2-hydroxy-	0.0	0.0
1-Amino-4-hydroxy-	0.0	0.0
2-Amino-3-hydroxy-	2.3	0.0
1-Amino-4-hydroxy-5-chloro-	0.0	0.0
2-Amino-1,3-dibromo-	0.0	0.0
1-Amino-2-hydroxyethoxy-4-hydroxy-	0.0	0.0
1,5-Diamino-2,4,6,8-tetrabromo-	0.0	0.0
1-Amino-2-sulpho-	3.6	6.2
1-Amino-2-carbonylchloride	7.7	4.4
1-Amino-2-sulpho-4-bromo-	7.5	7.0
1,5-Diamino-2-sulpho-	2.1	8.3
1-Amino-2-chloro-	0.0	0.0
1-Amino-2-sulpho-4-bromo-	7.0	7.0
2-Amino-3-chloro-	0.0	0.0
1-Amino-2,4-dibromo-	0.0	0.0
1-Amino-2-bromo-4-hydroxy-	0.0	0.0
1-Sulpho- (sodium salt)	11.3	7.4
2-Sulpho- (sodium salt)	11.0	7.3
1,6-Disulpho- (sodium salt)	—	12.6
1,7-Disulpho- (sodium salt)	19.5	12.6
1,8-Disulpho- (sodium salt)	14.5	12.7
2,6-Disulpho- (sodium salt)	17.7	14.8
2,7-Disulpho- (sodium salt)	19.0	15.1
1-Chloro-2-carboxy-	6.6	3.55
2-Carboxy-	4.8	0.0
1-Nitro-	0.0	0.0
4-Nitro-1,8-dichloro-	0.0	0.0
1-Nitro-2-methyl-	0.0	0.0
1-Nitro-5-sulpho-	7.3	6.8
1-Nitro-6-sulpho-	6.7	6.6
1-Nitro-8-sulpho-	8.0	7.1
1-Nitro-7-sulpho-	9.1 *	6.5
1-Nitro-2-carboxy-	6.6	5.1
1-Nitro-5-chloro-	0.0	0.0
1-Nitro-2-carbonylchloride	7.8	4.4
2-Sulphochloride	9.5	7.7
1,5-Disulphochloride	17.4	13.7
1,8-Disulphochloride	11.4	13.0
2,6-Disulphochloride	17.4	12.4
2,7-Disulphochloride	17.4	12.4
1-Bromo-	0.0	0.0
2-Bromo-	0.0	0.0
1,3-Dibromo-	0.0	0.0
1,5-Dibromo-	0.0	0.0
1,3,5,7-Tetrabromo-	0.0	0.0
Anthraquinone	0.0	0.0
2-Methyl-	0.0	0.0
Dianthrimide	0.0	0.0
4,4′-Diaminodianthrimide	0.0	0.0

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* Elongated spot.

TABLE 64

R_F VALUES OF SOME CHLOROANTHRAQUINONES

(J. FRANC AND M. WURST, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 657)

Solvent: 80% ethanol.
Paper: Whatman No. 1, impregnated with 30% liquid paraffin (b.p. 190–275°) in benzene; descending.
Detection: U.V. light; visible light.

Anthraquinone	R_F
1-Chloro-	0.49
2-Chloro-	0.33
1,4-Dichloro-	0.18
1,5-Dichloro-	0.41
1,6-Dichloro-	0.24
1,7-Dichloro-	0.28
1,8-Dichloro-	0.47
2,6-Dichloro-	0.02
2,7-Dichloro-	0.14

N.B. All have zero mobilities during electrophoresis in 3 N NH_4OH and in 1 N CH_3COOH.

TABLE 65

R_F VALUES OF PHENOLS AND RELATED COMPOUNDS

(W. E. HILLIS AND A. CARLE, *Biochem. J.*, 74 (1960) 607)

Solvents: S_1 = 6% aqueous acetic acid.
S_2 = Butan-1-ol–acetic acid–water (6:1:2, by vol.).
Paper: Whatman No. 1 or No. 3 (ascending).
Length of run: 11.5 in.
Temperature of run: 20°.
Detection: Visible light; U.V. light; U.V. light after NH_3 exposure; ferric chloride–potassium ferricyanide; diazotized *p*-nitraniline–sodium acetate; vanillin–HCl; KCN solution; sodium periodate and aniline; aniline–phthalic acid; phloroglucinol–HCl; bromocresol green.

Compound	R_F	
	S_1	S_2
Lignan*	0.00	0.97
Gallic acid	0.40	0.59
(+)-Catechin	0.40	0.59
(—)-*epi*Catechin	0.38	0.41
Ellagic acid	0.00	0.35
Corilagin	0.39	0.20
Juglanin	0.50	0.14
1-Galloyl-β-glucose	0.68	0.27
1,3,6-Trigalloylglucose	0.12	0.32
Shikimic acid	0.42	0.77
Leucodelphinidin	0.45	0.20

* Tentatively identified as a member of this group of substances.

TABLE 66

R_F VALUES OF SOME AMINO ACIDS

(H. E. WRIGHT, JR., W. W. BURTON AND R. C. BERRY, JR., *Arch. Biochem. Biophys.*, 86 (1960) 94)

Solvents: S_1 = *sec.*-Butanol–3% aqueous ammonia (5:2); run twice.
S_2 = *sec.*-Butanol–90% formic acid–water (15:3:2).
S_3 = *n*-Butanol–glacial acetic acid–water (12:3:5).
S_4 = *n*-Butanol–methyl ethyl ketone–conc. ammonium hydroxide–water (5:3:1:1).
Paper: Whatman No. 1 (ascending).
Time of run: 20 h.
Detection: Ninhydrin spray (0.2 g ninhydrin in 90 ml acetone, followed by addition of 10 ml glacial acetic acid).

Amino acid	R_F			
	S_1	S_2	S_3	S_4
Cystine	0.10	0.05	0.11	0.02
Lysine	0.17	0.15	0.16	0.08
Histidine	0.25	0.11	0.17	0.13
Arginine	0.18	0.19	0.19	0.05
Serine	0.23	0.28	0.25	0.11
Glycine	0.23	0.32	0.27	0.07
Aspartic acid	0.10	0.29	0.27	0.02
Glutamic acid	0.10	0.39	0.34	0.02
Threonine	0.28	0.36	0.33	0.28
Alanine	0.28	0.49	0.38	0.11
Proline	0.34	0.49	0.41	0.13
Tyrosine	0.41	0.52	0.47	0.17
Methionine	0.51	0.64	0.53	0.23
Valine	0.47	0.69	0.55	0.21
Tryptophan	0.59	0.54	0.55	0.32
Phenylalanine	0.66	0.70	0.64	0.35
Leucine	0.66	0.78	0.70	0.34
Isoleucine	0.61	0.77	0.68	0.31

TABLE 67

R_F VALUES OF SOME PHENYLALANINE DERIVATIVES

(Z. A. SHABAROVA, L. G. ANDRONOVA, M. BEZDEK AND M. A. PROKOFIEV, *Compt. rend. acad. sci. U.R.S.S.*, 130 (1960) 346)

Solvents: S_1 = *n*-Butanol saturated with water.
S_2 = *n*-Butanol–water–acetic acid (4:1:1).
Paper: Not stated.
Detection: Not stated.

Compound	R_F	
	S_1	S_2
N-(2′,3′-Isopropylidene-adenosine-5′-benzylphospho)-phenylalanine methyl ester	0.87	
N-(2′,3′-Isopropylidene-adenosine-5′-phospho)-phenylalanine	0.50	
Glycylphenylalanine methyl ester	0.42	
Valylphenylalanine methyl ester	0.69	
Tyrosylphenylalanine methyl ester		0.65

TABLE 68

R_F VALUES OF SOME SIMPLE PEPTIDES

(S. K. MAJUMDAR AND S. K. BOSE, *Biochem. J.*, 74 (1960) 596)

Solvents: S_1 = Water-saturated phenol.
S_2 = Butanol–acetic acid–water (4:1:1).
Paper: Whatman No. 1 (56 cm × 46 cm), descending.
Detection: 0.2 % ninhydrin in acetone.

*Peptide**	R_F S_1	R_F S_2
Asp·Glu	0.18	0.11
Ser·Asp	0.26	0.12
Glu·(Asp,Ala)	0.26	0.21
Glu·Asp	0.30	0.15
Asp·Ala	0.35	0.28
Asp·Tyr	0.36	0.34
Ala·Asp	0.40	0.40
Asp·(Glu,Tyr)	0.48	0.30
Glu·Tyr	0.50	0.41
Leu·(Asp,Glu)	0.52	0.36
Leu·(Asp,Glu,Ala)	0.60	0.42
Asp·(Ser, Tyr)	0.74	0.47
Ser·(Asp,Glu,Leu)	0.76	0.56
Ser·(Asp, Leu)	0.77	0.67
Ala·(Asp,Pro)	0.80	0.34

* Suggested structure. Tentative order given in brackets.

TABLE 69

ELECTROPHORETIC MOBILITIES OF ODONTOPHORE POLYSACCHARIDE AND SOME OTHER ACID POLYSACCHARIDES

(J. W. LASH AND M. W. WHITEHOUSE, *Biochem. J.*, 74 (1960) 351)

Electrolyte: 0.2 M sodium borate buffer pH 8.6.
Paper: Whatman No. 3 MM.
Potential: 12 V/cm.
Units: $\mu \times 10^{-5}$ cm^2/sec^{-1}/V^{-1} at 22°.
Detection: 0.15 % (w/v) thionin in 65 % (v/v) ethanol, acidified with approx. 0.1 % (v/v) acetic acid. Excess stain washed out either with 95 % (v/v) ethanol or running tap water.

Compound	*Sulphate content* (%)	*Mobility*
Odontophore polysaccharide	35	— 12.2
Chondroitin sulphate (sodium salt)	15	— 11.8
Heparin (sodium salt)	44	— 14.5

TABLE 70

R_F VALUES OF FATTY ACIDS AS THEIR 2,4-DINITROBENZYL ESTERS

(M. JUREČEK, J. CHURÁČEK AND V. ČERVINKA, *Mikrochim. Acta*, (1960) 102)

Paper: Whatman No. 1 impregnated with 25% formamide in ethanol.
Solvent: Cyclohexane–benzene (21:1).
Temperature: 21°.
Preparation of derivatives: the sodium salts of the acids are heated in a sealed tube for 1 h at 100° with the required amount of 2,4-dinitrobenzyl bromide.

Ester of	R_F
Formic acid	0.00
Acetic acid	0.13
Propionic acid	0.34
Butyric acid	0.56
Isobutyric acid	0.58
Valeric acid	0.73
Isovaleric acid	0.73
Caproic acid	0.81
2,4-Dinitrobenzyl bromide	0.35

TABLE 71

R_F VALUES OF SOME N-ALKYL-3,5-DINITROBENZAMIDES

(M. VEČEŘA, B. VOLÁKOVÁ, M. KOZÁKOVÁ AND M. JUREČEK, *Collection Czechoslov. Chem. Communs.*, 25 (1960) 1281)

Solvent: Benzene–cyclohexane (2:1).
Paper: Whatman No. 1.
Impregnation: With formamide; $I_1 = 10\%$, $I_2 = 20\%$, $I_3 = 30\%$.
Temperature of run: 22°.
Detection: 1% alcoholic α-naphthylamine, then U.V. observation; fresh acetone–2 *N* NaOH (1:1) spray.

Alkyl group	R_F		
	I_1	I_2	I_3
Methyl	0.16	0.10	0.05
Ethyl	0.27	0.23	0.13
n-Propyl	0.53	0.40	0.24
Isopropyl	0.51	0.36	0.22
n-Butyl	0.69	0.57	0.41
Isobutyl	0.66	0.52	0.38
sec.-Butyl	0.65	0.52	0.37
tert.-Butyl	0.78	0.68	0.54
n-Amyl	0.80	0.72	0.53
Isoamyl	0.78	0.68	0.50
n-Hexyl	0.85	0.82	0.70

TABLE 72

R_F VALUES OF ARTIFICIAL VANILLA FLAVOURS

(E. SUNDT, in *New Research Laboratories in Geneva*, Brochure of Firmenich & Cie, Geneva, 1959, p. 27)

Solvent: Cyclohexane–cyclohexene (5:3, v/v, previously saturated with dimethylformamide).
Paper: Whatman No. 7, impregnated with dimethylformamide.
Temperature: 25°.

Compound	R_F
Vanillin	0.06
p-Hydroxybenzaldehyde	0.09
Ethylvanillin	0.13
o-Vanillin	0.26
Veratraldehyde	0.27
Coumarin	0.29
Dihydrocoumarin	0.30
Piperonal (heliotropin)	0.35
6-Methylcoumarin	0.39
Propenylguaethol	0.44

TABLE 73

R_F VALUES OF SOME PHENOLS AND PHENOLIC ACIDS

(H. E. WRIGHT, JR., W. W. BURTON AND R. C. BERRY, JR., *Arch. Biochem. Biophys.*, 86 (1960) 94)

Solvents: S_1 = 2% Acetic acid.
S_2 = Sodium formate–90% formic acid–water (10 g:1:200).
S_3 = Isopropyl alcohol–conc. ammonium hydroxide–water (20:1:2).
S_4 = 8% Sodium chloride–acetic acid (100:1).
Paper: Whatman No. 1 (ascending).
Time of run: 4 h (aqueous solvent).
20 h (isopropanol solvent).
Detection: Diazotized *p*-nitraniline; diazotized sulphanilic acid.

Compound	R_F				*Colour**
	S_1	S_2	S_3	S_4	
Phloroglucinol	0.53	0.51	0.46	0.53	Orange
Salicylic acid	0.67	0.68	0.48	0.70	Yellow
m-Hydroxybenzoic acid	0.64	0.70	0.39	0.59	Rose red
p-Hydroxybenzoic acid	0.59	0.63	0.25	0.55	Rose red
p-Hydroxyphenylacetic acid	0.76	0.78	0.41	0.81	Violet
Protocatechuic acid	0.48	0.55	0.02	0.42	Violet
3,5-Dihydroxybenzoic acid	0.50	0.59	0.20	0.41	Yellow

* Colour of dye formed with diazotized *p*-nitraniline.

TABLE 74

R_F VALUES OF 2-NAPHTHYL SULPHAMATE AND 2-NAPHTHYLAMINE

(A. B. ROY, *Biochem. J.*, 74 (1960) 49)

Solvent: Butan-1-ol–propan-1-ol–aq. 0.1 *N* NH_3 (2:1:1, by vol.).
Paper: Whatman No. 1 (descending).
Detection: Ehrlich with heating to 37° for 30 min, successively sprayed with: *N* HCl; 0.1% (w/v) $NaNO_2$ and 0.5% (w/v) thymol in 2 *N* NaOH.

Compound	R_F
2-Naphthyl sulphamate	0.26
2-Naphthylamine	0.83

TABLE 75

R_F VALUES OF CYANIDIN CHLORIDE AND A METHYLATED PRODUCT

(W. G. C. FORSYTH AND J. B. ROBERTS, *Biochem. J.*, 74 (1960) 374)

Solvents: S_1 = Acetic acid–water–conc. hydrochloric acid (30:10:3).
S_2 = Butanol–2 *N* hydrochloric acid (1:1, v/v).
Paper: (a) not stated; presumably Whatman.
(b) Whatman seed test paper.
Detection: Ferric chloride–potassium ferricyanide; vanillin–conc. HCl; visible light.

Compound	R_F	
	S_1	S_2
Cyanidin chloride	0.50[a]	0.70[a]
3′,4′,5,7-Tetra-O-methylcyanidin		0.5–0.6[*][b]

[*] Circular paper chromatography.

TABLE 76

R_F VALUES OF SOME PORPHYRINS

(J. E. O'HAGAN, *Biochem. J.*, 74 (1960) 417)

Solvents: S_1 = Lutidine–water–NH_3 (NICHOLAS AND RIMINGTON, *Scand. J. Clin. Lab. Invest.*, 1 (1949) 12).
S_2 = Chloroform.
S_3 = Solvent of CHU, GREEN AND CHU, *J. Biol. Chem.*, 190 (1951) 643.
Paper: Not stated.
Temperature of run: 21°.
Detection: U.V. detection on solvent damp chromatograms.

Compounds	R_F		
	S_1	S_2	S_3
Aetiohaemin III	1.0	0.0	1.0
Dimethylprotohaemin IX	1.0		
Protohaemin[*]	0.7		
Tetramethylcoprohaemin III			0.63
Octamethyluroporphyrin I			0.21

[*] Tentative identification.

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List of Compounds Electrophoresed

GUIDE TO THE LIST OF COMPOUNDS ELECTROPHORESED

The List of compounds electrophoresed includes as many compounds as we were able to collect and identify. In addition, some items refer to "fragments" and "sub-units" of macromolecular entities, *e.g.*, proteins, which are the result of structural analysis. In this respect, as mentioned already in the Introduction, this Biblography differs from those previously published. As it frequently happens that naturally occuring macromolecular substances do not carry a specific name in the sense of chemical terminology, and as a close investigation of the papers involved appeared to us to be worth including in this book, we felt forced to list the substances subjected to electrophoresis under the group name with an additional specification of the source material.

Example: **Macroglobulin(s)**

–, α_1-
–, – from rabbit
–, α_2-
–, – bovine
–, – human
–, – – **subunits**
etc.

The reason is obviously that whole classes of natural compounds may be included under a single name, lactate dehydrogenase being a good example. In order to make the information more up-to-date, we have added the source in all instances in which it was possible to obtain the information from the original paper. In some papers, however, the authors themselves do not mention the source they used during their work. In such a situation, which occurs mostly with incompletely specified commercial preparations, we have included only the group name in the List of compounds electrophoresed.

In cases when the naturally occurring macromolecular compound was characterized by its molecular size in Svenberg units, this number is also added.

Example: **Glycoproteins**, 9,5 Sα_1

In general, the following types of compounds are not indexed here.

(1) Peptides without specific biological action, which are low-molecular-weight peptides resulting mainly from structural protein analyses. These are to be found under the parent compound with the addition "structural studies".
Example: **Actin**, structural studies

(2) Commercial names of preparations that are mixtures of several compounds, except mixtures of enzymes which have a widely used trivial name.
Example: **Pronase**

(3) Oligonucleotides, which arise as a product of the structural analysis of nucleic acids, are

omitted and are to be found under DNA and RNA, structural studies. The system of listing these is identical with that under item (1).

When preparing the List of compounds electrophoresed, we followed several rules, as listed below. Firstly, if there is a simple trivial name for a compound that has a complex chemical name, the former was preferred. This approach also holds for enzymes, which, when data were available, are supplied together with the corresponding E.C. number. The name of the enzyme according to the Enzyme Commission nomenclature is given whenever possible as a cross-reference. Problems of nomenclature such as the existence of different names for the same compound (globulins, for instance) can easily be a source of confusion; in order to avoid this unfavourable situation, it was decided to use cross-references whenever the need for them was felt. For example, for toxins cross-references to neurotoxins and venoms were added.

Under glycoproteins, only those which are commonly recognized as glycoproteins are listed. It happens, therefore, that other compounds which by definition can also be considered as glycoproteins, should be looked for under a specific name in another chapter of this book and in another part of this List of compounds electrophoresed.

As far as stereoisomers and optically active substances are concerned, they are not distinguished in the present List, except for enzymes (*e.g.*, see **L-Arabinose isomerase**), nucleosides and nucleotides (*e.g.*, see **Uracil**, 1-(5′-amino-5′-deoxy-β-D-**xylofuranosyl)-)**.

In sub-dividing a large series of compounds that differ according to their origin, involving procedures for isolating substances from natural material and in dealing with structural studies on these types of compounds, the following hierarchy (from which some items may, of course, be omitted) was used:

tRNA

– from . . . (different sources)
– from . . . (different sources), structural studies
– structural studies
– . . . . . . (different chemical modifications)
– – (different chemical modifications), from . . . (different sources)
– – structural studies

The system for organic compounds

In general, the system introduced by Macek and co-workers (Bibliography of Paper and Thin-Layer Chromatography 1961–1965, p. 828) has been followed throughout this work. In our opinion, this approach has the advantage of adequate uniformity within the bibliographic series. For those readers who do not possess the quoted Bibliography, these rules, together with our slight alterations, are repeated here. Trivial names have been preferred in selecting names for the compounds indexed, as it will be under these names that most readers will look for them. When several trivial names exist, the most commonly used name was chosen.

Substances frequently subjected to electrophoresis

The names of compounds subjected to electrophoresis relatively rarely are followed by numbers referring to all relevant papers in the bibliography. A reference to a whole chapter *in italics* is given for a compound, if most of the papers which deal with it are located in one chapter of the bibliography (*e.g.*, **Glycosaminoglycans**); in addition, references are also given to other papers describing the electrophoretic technique for the compound when these are located in other chapters.

Finally, the names of substances whose electrophoretic separation is described in a number of papers or in any book on electrophoresis, which may be consulted for the technique, are followed by a reference *in italics* marked with*, which indicates the chapter dealing with them (*e.g.*, a reference to the whole amino acid group is given at alanine). No more attention is paid to the occurrence of such a substance in other chapters. In view of the space available, it was not possible to include all synonyms – the book does not aim to be a substitute for a chemical dictionary.

The reader is recommended to note the following rules, which were followed in naming compounds for which no common trivial names exist:

(1) The names formed according to the substitution principle are given under the main part of the name. This part, placed first and printed in **bold** type, is called the "index compound", and is followed first by functional prefixes and then by endings, both in **alphabetical** order.

Examples: Dinitroaniline *see under* **Aniline**, 2,4-dinitro-
Dimethylmannose *see under* **Mannose, 3,4-di-O-methyl-**
Benzyloxymethyluracil *see under* **Uracil**, 5-benzyloxymethyl-

(2) The first separable functional ending following a name is considered to be part of the index compound and both together represent a single index heading.

Examples: 1-Amino-4-bromoanthraquinone-2-**sulfonic acid** *see under* **Anthraquinone-2-sulfonic acid,** 1-amino-4-bromo-;
similarly Propionic acid, 2-benzoylamino-3-methylamino, methylamide, etc.

(3) Names formed by combining a radical (or several radicals) and a designation of a functional class or a name of a cation or anion are used unchanged. Fusion-type names are treated in the same way.

Examples: **Ethylmorphine**
Methyl phosphorofluoridate

(4) When these radical-type names (*cf.*, the preceding point) are substituted, they are regarded as index compounds representing index headings.

Examples: **Ethylamine**, 1-phenyl-
Propylamine, 3-methylmercapto-

(5) For compounds with several different functional groups, the major function is determined from the following order of precedence for citation as a suffix: carboxylic acid, sulphonic acid, amide, aldehyde, nitrile, ketone, alcohol, phenol, thiol, amine, ether, thioether (sulphoxide, sulphone). Other functional groups are generally treated as suffixes.

Example: **Naphthol**, amino-, and not **Naphthylamine**, hydroxy-

(6) The prefixes oxa, thia and aza, as well as the condensation prefixes ending in -o (such as benzo- and naphtho-) and the prefixes thio- and seleno-, belong to the index compounds

and are therefore inseparable as prefixes. The same holds for the prefixes *allo*-, bis-, cyclo-, epi-, iso-, and nor-, which are considered in alphabetizing the names.

Examples: **Azathymidine**

Cyclopenten-3-one, 1-hydroxy-, **phosphate**

Norpantetheine, ethyl-, 4-monophosphate

2-Thio-6-azauridine

(7) The prefixes *n*-, *sec.*-, *tert.*-, *o*-, *m*- and *p*-, are italicized and have been ignored when alphabetizing. The prefixes N, O and S, etc., have also been ignored when alphabetizing.

(8) Deoxy-compounds have in most instances been entered under the name of the parent compound, *e.g.*, deoxyribose under **Ribose**, deoxy-, but some special compounds have been entered under their full names, *e.g.* **Deoxyribonucleic acid**.

(9) The cations and the majority of amphoteric compounds are indexed under the respective element. Only anions of elements of strongly acidic character are indexed as such (*e.g.*, for sulfates, *see* **Sulfates**).

A

Aabomycin 7741
Ablastamycin 7758
Acacetin 612
Acetaldehyde, sulfo- 7600
Acetal phosphatides, from human serum 1031
Acetate kinase (E.C. 2.7.2.1), from *Escherichia coli* 5625
Acetic acid 562, 8004
–, fluoro- 1003
Acetoacetyl coenzyme A thiolase (E.C. 2.3.1.9), from yeast 5534, 5535
Acetohydroxamic acid, N-carboxymethylimino-, iron complex 1400
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INTRODUCTION

During recent years, it has been the editorial policy of the Journal of Chromatography to supply its readers with extensive information about groups and types of substances separated. In this issue, a Bibliography on Electrophoretic Techniques is presented. As in previous Bibliographies, this one also had to respect the specific problems of the separation technique to which it is devoted.

This book is complementary to previously published Bibliographies on paper, thin-layer and column chromatography, and it may be attractive because of the vast development in electrophoretic techniques that is currently occurring and which is expected to increase in the near future. However, this development does not cover equally the technique itself and its routine applications. Many applications nowadays have become simply routine procedures applied in laboratories in the same way as classical analytical procedures such as titrimetry and gravimetry. Some of the electrophoretic techniques that exhibit molecular sieving effects, particularly polyacrylamide gel electrophoresis, have slowly replaced complicated and sophisticated systems and devices for the determination of molecular weight in everyday practice.

When starting with the selection of papers for this Bibliography, we had to take a decision on which classification system would be the most appropriate. As in previous Bibliographies and in the classification system of the Journal of Chromatography, we tried to avoid a substantial change in the basic 35-item classification system, as we considered it absolutely necessary to have reasonable uniformity throughout all of these compilations of bibliographic data. It is our opinion that some research workers may need information on a particular type of compound or series of compounds with respect to all types of separation procedures, *e.g.*, paper, thin-layer and column chromatography, and perhaps also electrophoresis.

On the other hand, everyone who becomes involved in electrophoretic techniques recognizes very rapidly that the increase in the number of papers published on this separation procedures is not in proportion in all the diverse fields of chemistry and biochemistry and recognizes also that biochemistry and the separation of hydrophilic substances are the most popular fields. It would be interesting to know not how many papers on enzymes and proteins deal with electrophoretic procedures, but to know those which do not deal with this technique. The result of the present situation is, of course, that chapters on peptides and proteins, structural studies on proteins, and enzymes contain more references than the remainder of the book. It could easily be predicted that in electrophoresis we shall not deal with compounds that are not ionizable; therefore, we do not have a single entry about the separation of hydrocarbons. However, there are fields in electrophoresis in which this technique could be applied and which still lack applications. The separations of oxo compounds, phenols and alkaloids are good examples.

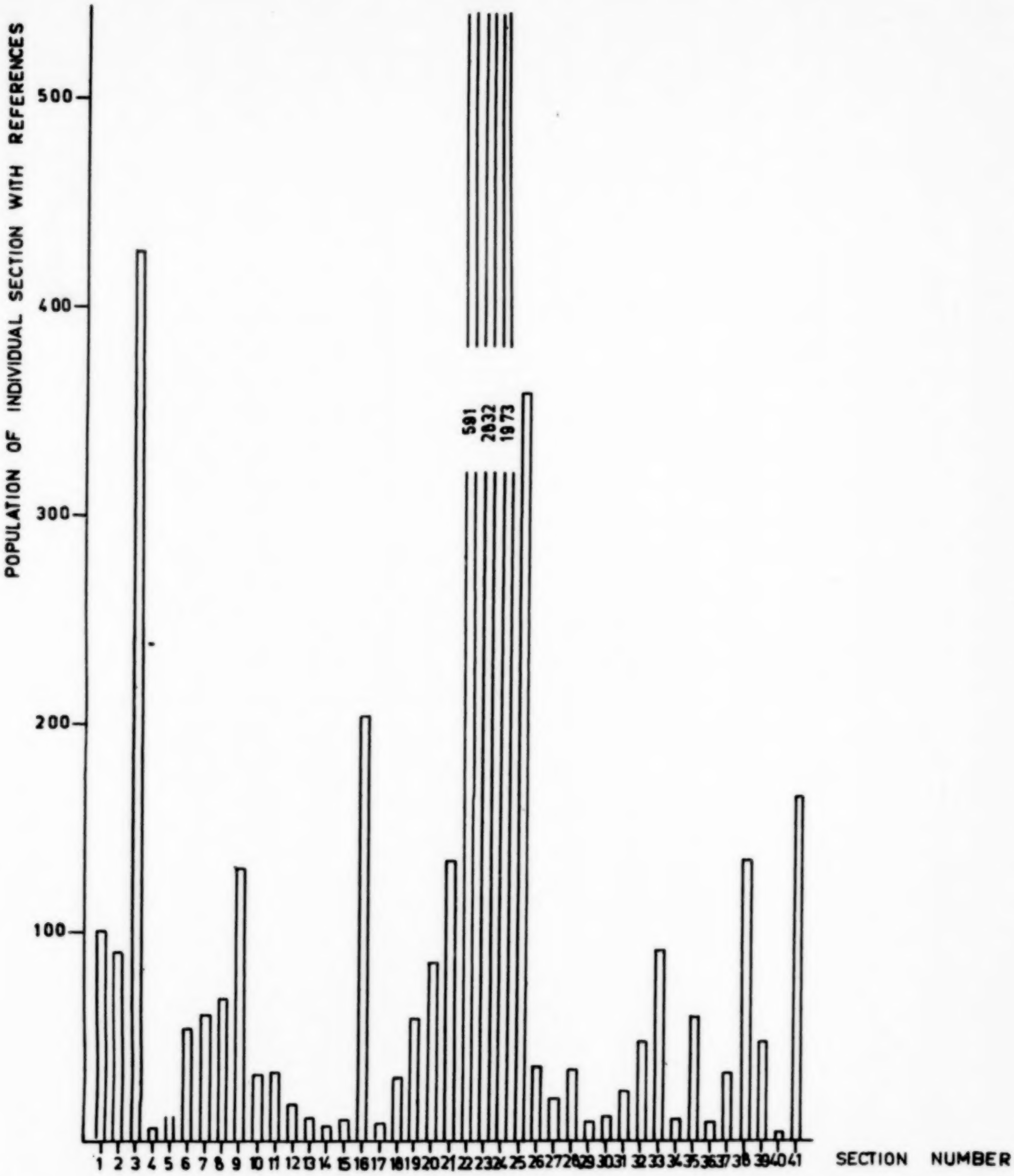

Fig. 1. Number of references to individual classes of compounds. The figure indicates the uneven application of electrophoretic techniques in different fields of chemistry. Item "Radioactive substances" does not involve all the separations in which radioactive compounds were used, but only those separations which are contributing to the technique of separating this particular type of compounds. Items "Structure investigation of polysaccharides and glycoproteins" and "Elucidation of primary structure of peptides and proteins" may, from another point of view, be transferred at least partly under "Peptides". 1 = Reviews and books; 2 = Theory; 3 = Technical progress; 4 = Alcohols and phenols; 5 = Oxo compounds and compounds containing heterocyclic oxygen; 6 = Carbohydrates; 7 = Carbohydrate derivatives; 8 = Glycosaminoglycans; 9 = Glycoproteins and glycopeptides; 10 = Structure investigation of polysaccharides and glycoproteins; 11 = Hemagglutinins; 12 = Carboxylic acids; 13 = Lipids; 14 = Glycolipids and lipopolysaccharides; 15 = Phospholipids; 16 = Lipoproteins; 17 = Steroids and terpenes; 18 = Amines and quaternary ammonium bases; 19 = Amino acids; 20 = Amino acid derivatives; 21 = Peptides; 22 = Elucidation of primary structure of peptides and proteins; 23 = Proteins; 24 = Enzymes; 25 = RNA; 26 = DNA; 27 = Alkaloids; 28 = Compounds with heterocyclic nitrogen; 29 = Organic sulphur and selenium compounds; 30 = Organic phosphorus compounds; 31 = Organometallic and coordination compounds; 32 = Vitamins; 33 = Antibiotics; 34 = Pesticides; 35 = Dyes and pigments; 36 = Macromolecular substances and plastics; 37 = Applications in pharmacology and toxicology; 38 = Inorganic compounds; 39 = Radioactive substances; 40 = Surfactants and detergents; 41 = Cells and subcellular particles.

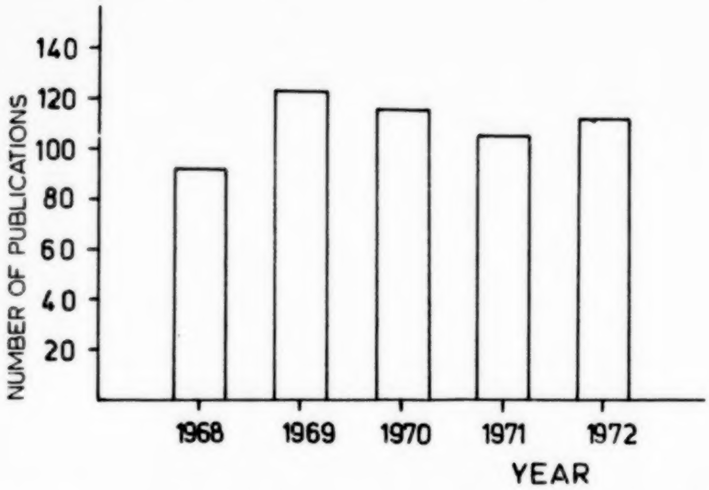

Fig. 2. Number of publications devoted to the methodical progress of electrophoresis during the years covered by this book. Papers summarized in "Applications" were not considered for this figure.

In the present volume, we have tried to supply the reader with information on both methodological progress and applications. As a substantial proportion of methodological progress is due to the vast expansion of electrophoretic separations in the area of proteins, enzymes and peptidic fragments and is applicable only in this area, we transferred these methodological papers to the sub-chapters General techniques in Proteins and in Enzymes. This might lead to a premature conclusion that there is less material on methodological progress than on applications. It is true, however, that in the years covered by the Bibliography (1968–1972), less has been published on methodology than on applications. We believe that the present Bibliography reflects very well the present situation in electrophoretic separations, with the exception of clinical and biological applications, which, if not contributing substantially to the separation procedures themselves, were omitted from this book. It can be concluded that it is the nature of the electrophoretic techniques used which makes the individual chapters of this book so uneven. This is demonstrated in Fig. 1, in which the population of individual chapters of this book with references is summarized. The publication of electrophoretic procedures during the period 1968–1972 is another fact which is worth illustrating. The result of our survey is presented in Fig. 2.

Rules for selecting papers

In general, the rules for selecting papers are identical in this Bibliography with those in Macek's Bibliography of Paper and Thin-Layer Chromatography and with those in our previous book on Column Chromatography. However, for those who do not have these books readily available, we would like to review these rules once more. Of publications which do not cover electrophoresis, we decided to take those which either present a new methodological aspect or which offer a general method of separation, isolation or purification of a particular group of compounds. This aspect has been applied even when the nature of the impurities present in the sample was unknown, but to a much lesser extent than in the Bibliography of Column Chromatography, as electrophoretic techniques are much less used for preparative purposes.

Electrophoretic techniques are frequently used for specific types of separations which are of high diagnostic value and widely used in clinical laboratories. This fact led us to introduce some special chapters throughout this book in which the biological, physiological and pathophysiological applications of certain types of electrophoretic separations are summarized. Also, the larger chapters had to be subdivided much more than was necessary in previous bibliographic volumes in order to be able to classify the references adequately. Of course, we are aware that our classification system for proteins is not perfect and that there could be a great deal of discussion about it. Also, in some instances it offers the possibility of listing a particular type of compound in two different chapters. The necessity to secure a certain degree of uniformity with the previous Bibliographies has been discussed already and, as we had to effect a compromise, we suggest that the reader who may not know where to look for a certain type of protein should consult the List of compounds subjected to electrophoresis at the end of this volume.

System of listing the references

In general, we utilized the experience gained during preparation of the previous Bibliographies, as we believe that from the practical point of view this system is adequate. With respect to the bibliography of electrophoresis published periodically in the Bibliography section of the Journal of Chromatography, we had to re-check every item, the result of which is naturally that some of the references have been listed under a different section from that in which it was originally placed, and some references are included which at first seemed unimportant from an electrophoretic point of view and which became interesting when investigated more closely. It also happened, although rarely, that other references were omitted for the same reasons. As mentioned before, we tried to keep the original 35-item listing system of the Journal of Chromatography and the previously introduced systems in the previous Bibliographies and, for the sake of clarity, we had to subdivide some of the original chapters, namely those which are related to high-molecular-weight substances, particularly proteins, peptides, enzymes and nucleic acids. On the other hand, because some chapters were much smaller, we had to combine them. These changes can easily be seen from the Contents.

We are also aware that various aspects of this volume could be subjected to some degree of improvement in future Bibliographies and in the bibliographic section which is published monthly in the Journal of Chromatography. We shall be obliged to anyone who is willing to consider the problems related to providing rapid information about separation procedures and to communicate with us.

Relation to other bibliographies

This Bibliography is naturally related to Bibliographies on other separation techniques, which have been published mainly in connection with the Journal of Chromatography and, to a much lesser extent, elsewhere. For those readers who are not familiar with these Bibliographies, they are summarized below:

I. Macek, K. and Hais, I.M.: *Bibliography of Paper Chromatography 1944–1956 and Survey of Applications.* Publ. House of the Czechoslovak Academy of Sciences, Prague, and Academic Press, London and New York, 1960, 767 pp. (Sold in Western countries with an English introduction.)

II. Macek, K., Hais, I.M., Gasparič, J., Kopecký, J. and Rábek, V.: *Bibliography of Paper Chromatography 1957–1960 and Survey of Applications.* Publ. House of the Czechoslovak Academy of Sciences, Prague, and Academic Press, London and New York, 1962, 706 pp.
Macek, K., Hais, I.M., Gasparič, J., Kopecký, J. and Rábek, V.: *Bibliographie 1957–1960 und Anwendungen.* Volume III of the serial publication: Hais, I.M. and Macek, K. (Editors): *Handbuch der Papierchromatographie.* G. Fischer Verlag, Jena, 1963, 700 pp.

III. Macek, K., Hais, I.M., Kopecký, J. and Gasparič, J.: *Bibliography of Paper and Thin-Layer Chromatography 1961–1965 and Survey of Applications* (The bibliography appeared as a Supplementary Volume of the Journal of Chromatography 1968).

IV. Macek, K., Hais, I.M., Kopecký, J., Rábek, V. and Churáček, J.: *Bibliography of Paper and Thin-Layer Chromatography 1966–1969 and Survey of Applications.* (The bibliography appeared as Supplementary Volume No. 2 to the Journal of Chromatography 1972).

V. Deyl, Z., Rosmus, J., Juřicová, M. and Kopecký, J.: *Bibliography of Column Chromatography 1967–1970 and Survey of Applications* (the bibliography appeared as Supplementary Volume No. 3 of the Journal of Chromatography 1973).

Formal notes

The headings of the articles are given in the original languages when these are English, German or French. When an abstract or summary of a paper is available in any of these languages, the authors have sometimes included its title. In other instances, the English translations of the titles have been given. Article headings are printed in parentheses if they are not given in the original languages. The brief characteristics that sometimes appear after the bibliographic data replace the title if the latter is inaccessible and serve to supplement it only when neither the title nor the location of the study in a certain chapter adequately reveals the exact nature of the electrophoretic problem involved.

Abstract journals. In the case of some studies published in the less accessible journals, we have also included the corresponding references in *C.A.*, *I.C.*, *Z. Anal. Chem.* or *Anal. Abstr.* The omission of such a reference, however, does not necessarily imply that the paper has not been abstracted in any of the above journals, and its inclusion, on the other hand, does not always mean that the original text was not available to the authors of the Bibliography. The article may therefore be referred to in the List of Compounds Electrophoresed even in the instances in which the particular compound is not mentioned in the abstract.

The transliteration of names of authors and titles of journals originally written in Cyrillic, Japanese or Chinese script has been carried out according to the style used in *C.A.* In some instances, when Russian or Bulgarian authors have published in Latin script, their non-standard transliteration may have been followed in the reference, but standard transliteration was used for the Author Index. Serbian names were transliterated according to the Croatian system.

Abbreviations of journal titles. In principle, we have used the abbreviations included in "Access", 1969 ed., a publication of the Chemical Abstracts Service published by the American Chemical Society.

Whenever two different types of listing of compounds are possible, preference is given to the chemical nature of the compound chromatographed rather than to the source or type of application. Thus haemoglobins are located among metalloproteins and not among blood proteins. If there are several substituents of equal importance in an organic compound, the compound is listed under the type which, according to the present listing system, comes later: thus a compound containing amino, nitro, oxo and thio groups is located under Organic sulphur compounds.

Most of the items listed carry a special note referring to the media in which the electrophoretic separation occurred. If a sorbent or medium not generally used is involved, a reference is given to the original paper in whcih the preparation of this medium has been described. However, in some items this information is missing, *e.g.*, for those papers which were available to us only as an abstract in which such details are not always given.

Papers in which the molecular weights of individual compounds separated were estimated by electrophoretic sieving techniques are either directly listed in the chapter Relation between molecular properties and electrophoretic behaviour, if they contribute substantially to the theory of electrophoretic separation according to molecular weight. If, however, the application to a specific type of compounds is more important, the paper is listed in the corresponding chapter in **Special Part** and a cross-reference to the abovementioned chapter is given.

Answers that can be supplied by the present bibliography:

(1) Which studies are concerned with a particular type of electrophoretic separation? What is the progress in the equipment? For what type of compounds is a particular type of electrophoretic separation applicable? These papers have to be searched for mainly in the General Part, with the exception of specialized applications such as electrophoresis and detection of proteins, peptides, nucleic acids and enzymes.

(2) Which studies are concerned with a particular group of substances? In order to find these papers, follow the Contents of the second part of the book (**Special Part**).

(3) Which studies deal with the particular compound or, alternatively, has a particular compound ever been subjected to electrophoresis? Check the List of Compounds Electrophoresed for this purpose. Compounds listed directly in the Contents of the **Special Part section are not included in the List of Compounds Electrophoresed.** Also, individual amino acids, nucleotides and sugar polymers are listed only under the group names, and it is therefore recommended that the Contents should be also checked for completeness. For more detailed information regarding the List of Compounds Electrophoresed, see p. 751. Because of the specific problems with proteins, in which a number of chemical entities are characterized just by a group name and source from which the particular protein has been isolated, the source material is included in the List of Compounds Electrophoresed.

(4) What papers have been published by a particular author? Check the Author Index for this purpose.

(5) What sorbents or what media have been used for a particular group of compounds? Check the notes attached to the end of individual items of the Bibliography and use

the Contents of the Special Part section for finding the appropriate group of compounds.

(6) Which compounds have been separated by electrophoresis side by side in a particular publication? As in the previous Bibliographies, this book also does not supply the reader with a direct answer. However, some information can be derived from the heading under which a particular paper is listed. In spite of the fact that the list of all the compounds separated in a particular paper has been available to us during the preparation of this publication, it has been omitted in the final stage in order to keep the Bibliography to a reasonable size.

This Bibliography covers over 90 journals known to publish papers on electrophoretic procedures. The time period covered is from January 1968 to December 1972. Of course, in addition to regular searches in journals, occasional references available to us directly as reprints to be found in the main chemical and abstract journals are included. The code for an abstract journal is supplied in this case: the main abstract journals checked for completeness are *C.A. (Chemical Abstracts), Anal. Abstr. (Analytical Abstracts)* and *I.C. (Index Chemicus).*

Papers that are not listed in this Bibliography include:

(1) papers that fail to show a substantial contribution to the technical development of electrophoresis;
(2) meeting reports and corresponding shortened versions (abstracts), even if they appeared in print;
(3) papers that were not available to us or that we failed to find.

To conclude this Introduction, we would like to say that we are aware that there must be mistakes in this book. We have come to the conclusion that it is practically impossible to avoid such errors. We worked on this Bibliography with maximum care and we sincerely hope that those mistakes which remain will not decrease the value of the information in this book. This also applies to this Introduction. From personal experience, I know that most readers do not read Introductions. I don't either! As a matter of fact, the only rare occasion on which I do read an Introduction is when I have to write one myself, and the only reason is to obtain some inspiration from people who are more qualified, more intelligent and more eloquent than myself. Therefore, I believe that if there is something missing from this Introduction which should have been mentioned, few will realize it and perhaps it will also not detract from the remainder of the book.

Prague, April 30th, 1973 Zdeněk Deyl

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General Part

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GENERAL REVIEWS AND BOOKS

Books on electrophoretic separations

1 Anonymous: *International Symposium V, Chromatographie Électrophorèse, Bruxelles, 1968,* Presses Académiques Européennes, 1969, 594 pp.

2 Anonymous: *International Symposium VI, Chromatographie Électrophorèse, Bruxelles, 1970,* Presses Académiques Européennes, 1971.

3 Arquembourg, P.C., Salvaggio, J.E. and Bickers, J.N.: *Primer of Immunoelectrophoresis.* Karger, Basel, 1970, 83 pp.

4 Backhausz, R.: *Immunodiffusion und Immunoelektrophorese: Grundlagen, Methoden und Ergebnisse.* Fischer, Jena, 1967, 516 pp.

5 Bier, M. (Editor): *Electrophoresis,* Vol. 2, Academic Press, New York, 1967, 533 pp.; *C.A.*, 68 (1968) 72695g.

6 Cann, J.R.: Electrophoresis. In Leach, S.J. (Editor): *Physical Principles in Technical Protein Chemistry.* Academic Press, New York, 1969, p. 369–450; *C.A.*, 72 (1970) 128732u.

7 Cawley, L.P.: *Electrophoresis and Immunoelectrophoresis.* Little and Brown, Boston, Mass., 1969, 360 pp.

8 Cawley, L.P., Chin, H.P., Epstein, E. and Kelsey, R.L.: *Clinical Electrophoresis.* Ann Arbor Sci. Publ., Ann Arbor, Mich., 1969, 160 pp.; *C.A.*, 73 (1970) 11318t.

9 Chin, H.P.: *Cellulose Acetate Electrophoresis – Techniques and Applications.* Ann Arbor Sci. Publ., London, 1970, 139 pp.

10 Gerritson, T. (Editor): *Modern Separation Methods of Macromolecules and Particles.* (Vol. 2 of *Progress in Separations and Purification*). Wiley-Interscience, New York, 1969, 250 pp.

11 Gordon, A.H.: *Electrophoresis of Proteins in Polyacrylamide and Starch Gels.* North-Holland, Amsterdam, 1969, 150 pp.

12 Haywood, B.J.: *Electrophoresis; Technical Application, a Bibliography of Abstracts.* Ann Arbor Sci. Publ., Ann Arbor, Mich., 1969, 440 pp.; *C.A.*, 72 (1970) 36185q.

13 Hjertén, S.: *Free Zone Electrophoresis.* Almqvist and Wiksell, Uppsala, 1967, 117 pp.; *C.A.*, 68 (1968) 6710g.

14 Korkisch, J.: *Modern Methods for the Separation of Rare Metal Ions.* Pergamon Press, Oxford, 1969.

15 Maurer, H.R.: *Disc Electrophoresis.* Ann Arbor Sci. Publ., Ann Arbor, Mich., 1970, 594 pp.

16 Maurer, H.R.: *Disk-Elektrophorese, Theorie und Praxis der diskontinuierlichen Polyakrylamidgel Elektrophorese.* De Gruyter, Berlin, 1968, 221 pp.

17 Sargent, J.R.: *Methods of Zone Electrophoresis,* 2nd ed., BDH, Poole, 1969, 118 pp.; *C.A.*, 72 (1970) 136821n.

18 Shaw, D.J.: *Electrophoresis.* Academic Press, New York, N.Y., 1969, 144 pp.

19 Sherma, J. and Zweig, G.: Paper Chromatography. In Zweig, G. and Whitaker, J.R. (Editors-in-Chief): *Paper Chromatography and Paper Electrophoresis.* Vol. II. Academic Press, New York, 1971, 614 pp.

20 Smith, I. (Editor): *Chromatographic and Electrophoretic Techniques. Vol. 2. Zone Electrophoresis.* Interscience, New York, 2nd ed., 1968, 524 pp.; *C.A.*, 69 (1968) 39037s.

21 Smith, I.: *Chromatographic and Electrophoretic Techniques.* Wiley, New York, 1969, 1080 pp.

22 Stensio, K.E. and Ekedahl, G.: *Chromatography and Electrophoresis.* Norstedt, Stockholm, 1969, 227 pp.; *C.A.*, 73 (1970) 29334e.

23 Vigyazo, L.V.: *(Paper Electrophoresis).* Muszaki Kiado, Budapest, 1967, 288 pp.; *C.A.*, 69 (1968) 99828k.

24 Zweig, G. and Whitaker, J.R.: *Paper Chromatography and Electrophoresis.* Vol. I and II. Academic Press, New York, London, 1967.

Reviews on electrophoresis

Check also the chapters on Reviews which appear in the individual paragraphs of the Special Part for completeness.

25 Bayles, J.W.: Review of the physical chemistry of electrophoresis. *J. Oil Colour Chem. Ass.*, 55 (1972) 467–479; *C.A.*, 77 (1972) 52608p.

26 Brewer, J.M. and Ashworth, R.B.: Disc electrophoresis. *J. Chem. Educ.*, 46 (1969) 41–45 – polyacrylamide gel.

27 Cann, R.: Recent advances in the theory and practice of electrophoresis. *Immunochemistry,* 5 (1968) 107–134; *C.A.*, 68 (1968) 75588k.

28 Catsimpoolas, N.: Immunoelectrofocusing. *Sci. Tools,* 16 (1969) 1–5; *C.A.*, 71 (1969) 120361f – agarose gel, polyacrylamide gel.

29 Cirje, M., Boerescu, J. and Vacariu, A.: (Disc electrophoresis). *Stud. Cercet. Fiziol.*, 15 (1970) 349–355; *C.A.*, 74 (1971) 10164y.

30 Daussant, J. and Grabar, P.: L'analyse immuno-électrophorétique et ses domaines d'application. *Int. Symp. V, Chromatogr. Electrophor., Bruxelles, 1968,* 1969, p. 20–31.

31 Degraef, R. and Van Aert, A.: (Acrylamide gel disc electrophoresis). *Ind. Chim. Belge,* 36 (1971) 679–688; *C.A.*, 76 (1972) 6948x – polyacrylamide gel.

32 Everaerts, F.M.: Isotachophoresis. *J. Chromatogr.*, 65 (1972) 3–17.

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TECHNIQUES OF ELECTROPHORESIS

Laboratory devices for electrophoresis

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APPLICATORS AND PIPETTING DEVICES

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See also the chapters on Glycoproteins, glycopeptides and proteoglycans from connective tissue (877–899) and on Structure investigation of glycoproteins, proteoglycans and glycosaminoglycans (943–961) for completeness.

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STRUCTURE INVESTIGATION OF POLYSACCHARIDES AND GLYCOPROTEINS

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LIPOPROTEINS FROM HUMAN BLOOD

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AMINES AND QUATERNARY AMMONIUM BASES

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Applications

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See also: 1702.

ELUCIDATION OF THE PRIMARY STRUCTURE OF PEPTIDES AND PROTEINS

Cross-references to parent proteins are not supplied. Check the appropriate chapter among Proteins (2152–4983) for completeness.

Structural studies on microbial and plant proteins (including cytochromes and ferredoxin)

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Structural studies on invertebrate proteins

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Structural studies on vertebrate proteins

STRUCTURAL STUDIES ON PROTEINS OF BLOOD, PLASMA, SERUM AND BLOOD CELLS (EXCEPT HEMOGLOBINS)

Structure of immunoglobulins

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Structural studies on isomerases and ligases

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PROTEINS

For glycoproteins and lipoproteins see the chapters on Glycoproteins and glycopeptides (801–930) and on Lipoproteins (1035–1238), respectively.

Cross references to the chapter Elucidation of the primary structure of peptides and proteins (1561–2151) are not supplied.

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Applications

PROTEINS OF CELLS, VIRUSES AND SUBCELLULAR PARTICLES (EXCEPT BLOOD CELLS AND PLATELETS)

For proteins of chloroplasts see the chapter on Other proteins of plant origin (including plant histones, concanavalin and proteins of chloroplasts (2883–2969)).

Proteins of ribosomes

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MICROBIAL AND PLANT PROTEINS

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See also: 968, 975, 1224, 2162, 2197, 2393, 2446, 2447, 2473, 2478, 2583, 2588, 2683, 2718, 3869.

INVERTEBRATE PROTEINS

Invertebrate blood and hemolymph proteins (including invertebrate hemoglobins)

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See also: 2982, 5331.

VERTEBRATE PROTEINS

Proteins of blood, serum and blood cells (except hemoglobins)

Immunoglobulins

For immunoglobulins from other sources than blood see the respective chapters. Fundamental cross-references are supplied in this chapter.

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Fibrinogen, fibrin, thrombin and clotting related proteins

For invertebrate fibrinogen see the chapter on Invertebrate blood and hemolymph proteins (including invertebrate hemoglobins) (2970–3011).

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See also: 3440, 4534, 6411, 6429, 8202, 8203.

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See also: 6, 46, 78, 81, 85, 89, 2194, 2238, 3170, 3187.

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See also: 56, 776, 877, 882, 890, 2153.

Protamines, histones and other nuclear proteins

For histones of plant origin see the chapter on Other proteins of plant origin (including plant histones, concanavalin and proteins of chloroplasts) (2883–2969). For histones of microbial origin see the chapter on Other proteins of microbial origin (including flagellins, thioredoxin and histones of microbial origin) (2819–2882). For invertebrate histones see the chapter on Invertebrate basic proteins and histones (3032–3045).

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See also: 173, 174, 355, 390, 393, 773, 2268, 2317, 2882, 3039, 3045, 7155.

Chromoproteins and metalloproteins

For invertebrate cytochromes see the chapter on Other proteins of invertebrates (including cytochromes and silk proteins) (3046–3090). For microbial and plant cytochromes see the chapter on Metalloproteins and cytochromes of bacterial and plant origin (2649–2680).

Hemoglobin

For invertebrate hemoglobins see Invertebrate blood and hemolymph proteins (including invertebrate hemoglobins) (2970–3011).

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Transferrin, ferritin and ferredoxin

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See also: 166, 595, 2236, 2427, 2503, 3416, 4147, 4210, 4453, 4649, 5920, 6698, 6717.

Ceruloplasmin

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Proteins of brain, cerebrospinal fluid and eyes

Brain proteins (except pituitary hormones, including encephalitogenic protein)

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See also: 907, 2444, 2451, 2468, 2480, 2481, 3806, 3859, 3885, 4137–4139, 4827, 4919.

Proteins of cerebrospinal fluid and spinal cord

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See also: 2211, 2585.

Lens and eye proteins

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See also: 83.

Proteins of glands, gland products (except mammary gland) and various zymogens

For sweat proteins see the chapter on other proteins of gland origin (4531–4542). For peptidic and proteinous hormones see the respective chapter (4643–4814).

Pepsinogen, trypsinogen, chymotrypsinogen, other zymogens and related inhibitors (including α_1-antitrypsin)

For plasminogen see this chapter. For trypsin inhibitor in colostrum see the chapter on Complex mixtures and other milk proteins (including trypsin inhibitors from milk) (4612–4642).

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See also: 162, 174, 178, 856, 2236, 2277, 2503, 2543, 3416, 3511, 3575, 3576, 3658, 3659, 3665, 4496, 4498, 4649, 4918, 4998, 5447, 5699, 5859, 5920, 6357, 6381.

Snake venoms

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See also: 802, 4516, 4517, 4522, 6449, 6459, 6467.

Proteins of gonadal and related secretions

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See also: 409, 438, 550, 1867, 3120, 3129, 4556.

Complex mixtures and other milk proteins (including trypsin inhibitors from milk)

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See also: 3230, 3471, 5551.

PEPTIDIC AND PROTEINOUS HORMONES

For synthetic analogues and their fragments see the respective paragraph in the chapter on Peptides (1429–1560). Check also the chapter on Peptides in animal material (1537–1560) for completeness.

Pituitary hormones (including products of the pineal gland)

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See also: 56, 414, 596, 1554, 1924, 1925, 4196, 8058.

Thyroglobulins

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See also: 162, 169, 2309, 2543, 4649, 4715, 5817.

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See also: 443, 4537, 6374, 8054.

PROTEINS OF OTHER VERTEBRATE TISSUES

Liver proteins

This chapter includes unspecified mixtures of liver proteins. For defined proteins of liver origin see the specific chapters under Vertebrate proteins (3091–4642).

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See also: 598, 2589, 2593, 3508, 3534, 3609, 3796, 3797, 3806, 3807, 3816, 3825, 3837, 3851, 3857, 3866, 3873, 3880, 3886, 3888, 3896, 3900, 3908, 4137, 4859, 4900, 4907, 4949, 4976, 7388, 8184, 8232, 8236.

Lung proteins

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Epidermal and hide proteins and proteins of epidermal derivatives (except collagen, amyloid and elastin)

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See also: 4932.

MIXTURES OF OXIDOREDUCTASES AND UNCOMPLETELY SPECIFIED OXIDOREDUCTASES

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See also: 5272, 5338.

Transferases

TRANSFERRING ONE-CARBON GROUPS (METHYL-, HYDROXYMETHYL-, FORMYL-, CARBONYL-, CARBAMOYL-, AMIDINO- AND RELATED TRANSFERASES) (E.C. 2.1.-.-)

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TRANSFERRING ALDEHYDIC OR KETONIC GROUPS (E.C. 2.2.-.-.)

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See also: 5259.

ACYLTRANSFERASES (ACYL- AND AMINOACYLTRANSFERASES) (E.C. 2.3.-.-.)

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GLYCOSYLTRANSFERASES (HEXOSYL- AND PENTOSYLTRANSFERASES) (E.C. 2.4.-.-.)

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TRANSFERRING NITROGENOUS GROUPS (AMINOTRANSFERASES, OXIMINOTRANSFERASES) (E.C. 2.6.-.-.)

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See also: 4378, 4482.

TRANSFERRING PHOSPHORUS-CONTAINING GROUPS (E.C. 2.7.-.-) FROM MICROBIAL AND PLANT MATERIAL

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See also: 6805, 7088.

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Hydrolases

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See also: 2770, 2901, 2902, 7088.

ACTING ON ESTER BONDS (E.C. 3.1.-.-.) FROM ANIMAL MATERIAL

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See also: 2999, 3000, 3071, 3544, 3588, 3627, 3701, 4470, 4482, 5295, 5862–5864, 5872–5875, 5879.

ACTING ON GLYCOSYL COMPOUNDS (E.C.3.2.-.-.)
FROM MICROBIAL AND PLANT MATERIAL

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See also: 2940, 5858, 5866, 5867.

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ACTING ON PEPTIDE BONDS (E.C.3.4.-.-.) FROM VARIOUS MOSTLY ANIMAL TISSUES

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See also: 166, 2203, 4416, 4418, 4420, 4421, 4424, 4429, 4435, 4649.

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Lyases

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OTHER LYASES AND INCOMPLETELY IDENTIFIED LYASES (E.C.4.4.-.-., E.C.4.5.-.-. AND E.C.4.99.-.-.)

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See also: 2441, 5396.

CHEMICALLY MODIFIED PROTEINS AND ENZYMES

See: 166, 169, 178, 921, 1706, 1810, 1815, 1825, 1889, 1935, 1943, 1967, 1968, 1986, 1989, 1993, 1998, 2047, 2125, 2126, 2201, 2202, 2240, 2259, 2464, 2526, 2571, 2575, 2790, 3018, 3097, 3186, 3200, 3268, 3292, 3293, 3295, 3297, 3309, 3314, 3387, 3502, 3650, 3729, 3759, 3923, 3990, 4025, 4112, 4151, 4160, 4295, 4299, 4301, 4305a, 4307, 4359, 4428, 4440, 4448, 4449, 4552, 4589, 4594, 4599, 4654, 4657, 4659, 4672, 4674, 4675, 4729, 4763, 4765–4770, 4772, 4773, 4778, 4814, 4843, 5262, 5819, 5830, 5840, 5878, 6048, 6752.

NUCLEOTIDES, NUCLEOSIDES AND NITROGENOUS CONSTITUENTS OF NUCLEIC ACIDS

Some overlapping between individual parts of this chapter cannot be avoided. Check always related chapters for completeness. Check also the chapters on Structural studies of RNA (including total hydrolysates) (7398–7486) and Structural studies on DNA (including total hydrolysates) (7530–7535) for completeness.

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See also: 165, 172, 321, 345, 378, 8060, 8206.

Applications

SYNTHETIC APPLICATIONS

Purine nucleotides and nucleosides

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Pyrimidine nucleotides and nucleosides

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Amino acid nucleotide conjugates

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Products of chemical modification of naturally occuring nucleotides and nucleosides

(Modification involves the base only and does not refer to the carbohydrate part of the nucleotide or nucleoside.)

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See also: 7111, 7113, 7131.

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See also: 5630, 5825, 7299, 7487–7491,7494, 7495, 7499–7501, 7503, 7508, 7509, 8127.

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See also: 5808, 7492, 7493, 7497, 7502, 7504–7507.

STRUCTURAL STUDIES ON RNA (INCLUDING TOTAL HYDROLYSATES)

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DNA

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See also: 10.

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See also: 82, 562, 1272, 6972, 7544, 7550, 7559, 8044.

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Cations

The term cation is used in its usual analytical meaning irrespective of the fact that during electrophoresis it might occur in the form of a complex anion. For metal complexes check also the chapters on Metal complexes of amino acids (1399–1402) and Organometallic and coordination compounds (7617–7642).

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See also: 63, 88, 493, 549, 562.

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See also: 7957, 7958, 7961, 7992, 7994, 7995, 7997, 7998, 8039, 8064.

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